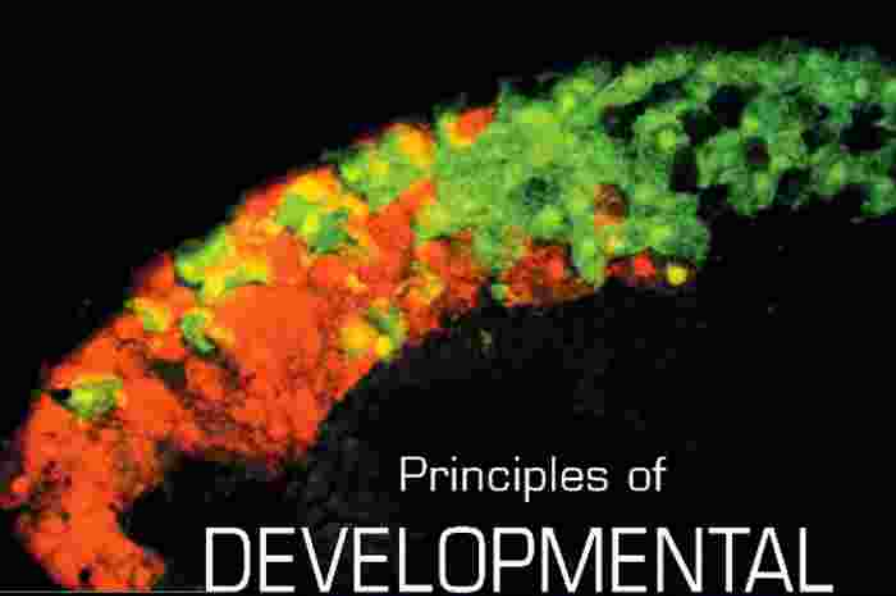

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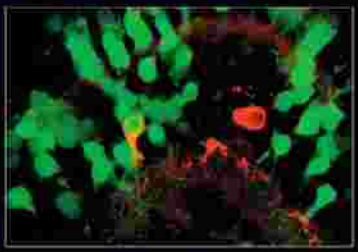

Sally A Moody

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PRINCIPLES OF DEVELOPMENTAL GENETICS

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PRINCIPLES OF DEVELOPMENTAL GENETICS

SALLY A. MOODY
George Washington University

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PREFACE

Developmental Genetics, or What Can Genetics and Genomics Tell Us About Evolution, Development, Stem Cells, Human Birth Defects, and Disease?

Sally A. Moody

Department of Anatomy and Cell Biology, George Washington University

The ability of researchers to answer experimental questions greatly depends on the available technologies. New technologies lead to novel observations and field-changing discoveries and influence the types of questions that can be asked. Today's recently available technologies include sequencing and analyzing the genomes of human and model organisms, genome-wide expression profiling, and high-throughput genomic and genetic analyses. The information provided by these approaches is enabling us to begin to understand the complexity of many biological processes through the elucidation of gene regulatory networks, signaling pathway networks, and epigenetic modifications. This book describes many lines of research that are being impacting by these new technologies, including developmental genetics and the related fields of clinical genetics, birth defects research, stem cell biology, regenerative medicine, and evolutionary biology.

The field of developmental genetics, or the study of how genes influence the developmental processes of an organism, has been influenced by new technologies and by interactions with other fields of study throughout its history. The concept of a genetic basis of development began in "modern" times at the intersection of descriptive embryology and cytology. Modern histological techniques were developed in the mid-19th century, largely by Wilhelm His so that he could study cell division in the neural tube, which enabled visualization of the cell nucleus, chromosomes, and the discrete steps of mitosis. Theodor Boveri cleverly applied these improved microscopic techniques to transparent marine embryos to demonstrate that each parent contributes equivalent groups of chromosomes to the zygote, and that each chromosome is an independently inherited unit. Importantly, he noted that if an embryo contains the incorrect number or improper combination of chromosomes, it develops abnormally.

However, many early embryologists rejected the idea that development is driven by prepackaged heritable particles because it seemed too similar to the idea of "preformation": the concept that development is driven by predetermined factors or "forces" (sometimes described in rather mystical terms).

Wilhelm Roux, an advocate of studying the embryo from a mechanistic point of view, was a leader in the approach of manipulating the embryo with microsurgical techniques to elucidate cause and effects between component parts (experimental embryology). By using an animal model whose embryos were large, developed external to the mother, could be surgically manipulated with sharpened forceps and cultured in simple salt media (i.e., amphibians), he rejected the role of predetermined factors and demonstrated the importance of external (epigenetic) influences and cell–cell interactions in regulating developmental programs. Experimental embryologists further refined their skills at dissecting small bits of tissue from the embryo, recombining them with other tissues in culture or transplanting them to ectopic regions in the embryo. This work led to the invention of tissue culture by Ross Harrison and the discovery of tissue inductions by Hans Spemann.

While experimental embryology was thriving, T. H. Morgan founded the field of Drosophila genetics. Also trained as an embryologist, Morgan was skeptical of Boveri's idea of heritable packets, and directed his studies towards understanding the principles of inheritance. For several decades, the two fields had little impact on one another. Interestingly, however, after a few decades of study of the fruit fly, Morgan's work supported the idea of discrete intracellular particles that directed heritable traits, which he named "genes." Nonetheless, the fields of experimental embryology and genetics remained fairly separate entities with distinct goals and points of view. Embryologists were elucidating the interactions that are important for the development of numerous tissues and organs, whereas geneticists were focused on the fundamentals of gene inheritance, regulation of expression, and discovering the genetic code. Indeed, elucidating the genetic basis of vertebrate development was delayed until new technologies in molecular biology and cloning were devised. From the field of bacterial and viral genetics came the techniques for cloning eukaryotic genes and constructing vectors for controlling expression. From the classical genetic studies in fly and nematode came the rationale for mutagenizing the entire genome and screening for developmental abnormalities. Important regulatory genes were discovered in these invertebrates, and their counterparts were discovered in many other animals by homology cloning approaches. Thus was born the modern field that we call developmental genetics.

An important advance in the past decade is the demonstration that genes that regulate developmental processes in invertebrate species have important developmental functions in vertebrates. The wealth of information concerning the molecular genetic processes that regulate development in various animals demonstrates that developmental programs and biological processes are highly conserved, albeit not identical, from yeast to human. Indeed, the Human Genome Project has made it possible to identify the homologues in humans and demonstrate that many of these regulatory genes underlie human developmental disorders and aspects of adult diseases in which differentiation processes go awry. Currently, researchers are studying the fundamentals of developmental processes in the appropriate animal model and screening humans for mutations in the genes identified by the basic research to be likely causative candidates. Researchers are mutagenizing vertebrate animal models and screening for mutants that resemble known human syndromes. This cross-fertilization of fields is also impacting concepts in evolutionary biology,

leading to a better understanding of "ancestral" species via gene expression profiles, and paradigms in stem cell biology in which naïve cells may be directed to "designer" lineages.

Most recently, there have been significant technological advances in genetic, genomic, and protein expression analyses that are having a major impact on experimental approaches and analytic design. The intersection of developmental biology with these technologies offers a new view of developmental genetics that is only beginning to be exploited. It is this new intersection at the onset of the genomic era that is the focus of this book. The book is organized into sections focused on different aspects of developmental genetics. Section I discusses the impact of new genetic and genomic technologies on development, stem cell biology, evolutionary biology, and understanding human birth defects. Section II discusses several major events in early embryogenesis, fate determination, and patterning, including cellular determinants (Boveri revisited?), gene cascades regulating embryonic axis formation, signaling molecules and transcription factors that regulate pattern formation, and the induction of the primary germ layers (ectoderm, mesoderm, and endoderm). Section III describes the reorganization of the embryo via different types of morphogenetic and cellular movements that result in the foundation of organ systems, and discusses the many signaling and adhesion molecules that are involved in regulating these complex processes. The final three sections focus on the signaling cascades and transcriptional pathways that regulate organogenesis in representative systems derived from the embryonic ectoderm, mesoderm, and endoderm. These chapters illustrate how embryonic rudiments become organized into adult tissues, and how defects in these processes can result in congenital defects or disease. Each chapter demonstrates the usefulness of studying model organisms and discusses how this information applies to normal human development and clinical disorders. Several chapters also discuss the utility of stem cells to repair damaged organs and the application of developmental genetics to the manipulation of stem cells for regenerative medicine.

The goal of this book is to provide a resource for understanding the critical embryonic and prenatal developmental processes that are fundamental to the normal development of animals, including humans. It highlights new technologies to be used, new questions to be answered, and the important roles that invertebrate and vertebrate animal models have had in elucidating the genetic basis of human development. Developmental genetics has reemerged from its birth a century ago as a nexus of diverse fields that are using the common language of gene sequence and function. This is influencing what questions are posed and how the answers are used. New technologies are making it relatively easy to study gene expression and regulation at single cell, tissue, and embryonic levels. The conservation between the genomes of species that are separated by vast evolutionary time encourages us to more fully utilize animal models to gain important insights into the clinical relevance of the animal model data. It is our hope that this book will stimulate even more cross-fertilization and interactions between evolutionary biology, developmental biology, stem cell biology, basic scientists, and clinical scientists.

I wish to thank all of the authors for contributing such exciting and excellent chapters, and Pat Gonzalez for keeping all of us on schedule.

RECOMMENDED RESOURCES

Baltzer F: *Theodor Boveri, life and work of a great biologist*, Los Angeles, CA, 1967, University of California Press.

Hamburger V: *The heritage of experimental embryology: Hans Spemann and the organizer*, New York, 1988, Oxford University Press.

Model Organisms for Biomedical Research (Web site): http://www.nih.gov/science/models.

Morgan TH: *The theory of the gene*, New Haven, CT, 1926, Yale University Press.

Spemann H: *Embryonic development and induction*, New York, 1967, Hafner Publishing Co.

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I

THE IMPACT OF GENETIC AND GENOMIC TOOLS ON DEVELOPMENTAL BIOLOGY

1 UNTANGLING THE GORDIAN KNOT: CELL SIGNALING EVENTS THAT INSTRUCT DEVELOPMENT

RENÉE V. HOCH and PHILIPPE SORIANO

Program in Developmental Biology and Division of Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, WA

INTRODUCTION

The developmental cell signaling field has evolved out of convergent work in developmental genetics and biochemistry. Landmark studies were performed during the 1980s and 1990s, when genetic screens identified mutants that enhanced or suppressed receptor tyrosine kinase (RTK) loss-of-function phenotypes in *Drosophila melanogaster* (*sevenless, torso*) and *Caenorhabditis elegans* (*Egfr*) embryos. Such mutants were arranged into hierarchies on the basis of epistatic relationships and cell-autonomous versus cell-nonautonomous effects on RTK functions (reviewed in Furriols and Casanova, 2003; Moghal and Sternberg, 2003; Shilo, 2003; Nagaraj and Banerjee, 2004). Concurrently, biochemical experiments validated the results of these screens and explored the molecular mechanisms underlying the observed genetic interactions. Thus, genetically defined hierarchies were translated into a molecular signal transduction cascade connecting RTKs to the activation of mitogen-activated protein kinase (MAPK; Figure 1.1, A; reviewed in Porter and Vaillancourt, 1998; Schlessinger, 2000). These efforts collectively demonstrated that RTKs signal through an evolutionarily conserved biochemical pathway that is required during development and that includes several proteins previously implicated in growth and oncogenesis.

The RTK studies set the stage both conceptually and experimentally for subsequent studies of cell–cell signaling. Similar approaches have subsequently identified and characterized the components of several pathways that are activated by cell–cell contact and/or secreted molecules, and mutant phenotypes in model systems have revealed their developmental roles. From these studies, we now know that major developmental signaling pathways such as

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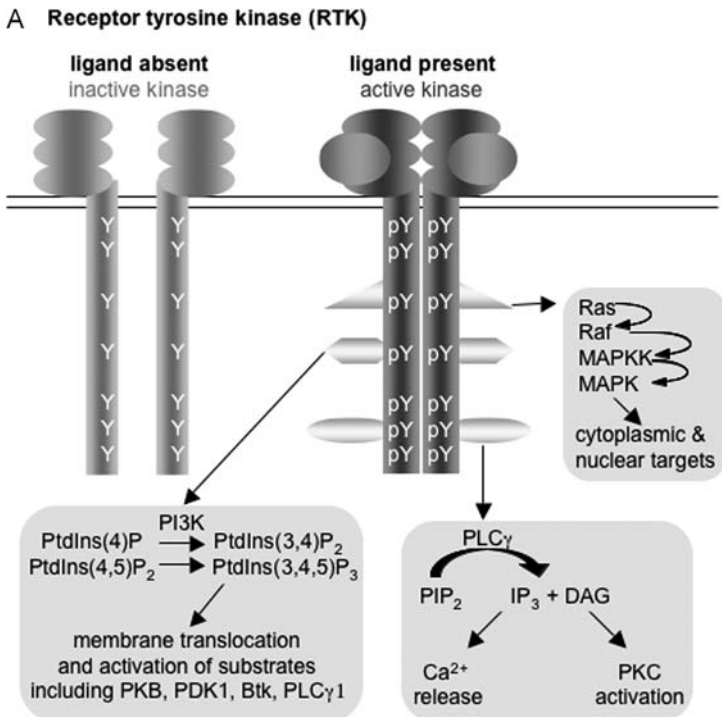

FIGURE 1.1 **Basic overview of the major cell–cell signaling pathways discussed in this chapter.** **A,** Receptor tyrosine kinases (RTK): Extracellular ligand (*red*) binds the receptor and, often by facilitating dimerization, induces the activation of cytoplasmic kinase domains. The receptors then autophosphorylate on several tyrosine residues, generating docking sites for effector proteins (*yellow*). Effector proteins initiate various signal transduction pathways when engaged by the receptors at the plasma membrane. Three pathways commonly activated by RTKs are shown, although members of the superfamily differ in their effector pathway usage and regulation. **B,** Hedgehog (Hh): In the absence of ligand, the Hh receptor Patched (Ptc) inhibits the Smo-initiated signaling pathway. In this state, Costal-2 (Cos2), Fused (Fu), and Gli/Ci form a complex, and Gli/Ci is preferentially proteolyzed to a repressive form (Gli^R) that translocates to the nucleus and blocks transcription. When Hh binds Ptc, Smo inhibition is relieved. Smo localizes to cilia (vertebrates) or clusters at the plasma membrane (invertebrates), is phosphorylated, and binds the Cos2–Fu complex. This releases Gli/Ci, which is then preferentially processed to a different product, Gli^A, that enters the nucleus and activates target gene transcription. **C,** TGFβ/BMP: Ligand binding to the heterotetrameric Activin receptor induces the type II subunits of this complex to serine/threonine phosphorylate the type I subunits, which then phosphorylate associated Receptor-Smads (R-Smads). SARA facilitates the interaction between R-Smad and the receptor. Phosphorylation of R-Smads increases their affinity for co-Smads and decreases their affinity for SARA, which is then released. Heterotrimers (R-Smad, co-Smad) or homotrimers (R-Smad) then form and translocate to the nucleus, where they regulate the transcription of target genes with help from DNA-binding cofactors and transcriptional coactivators or corepressors. **D,** Wnt/Wingless (Wg)–β-catenin pathway: In the absence of ligand, a destruction complex comprised of GSK3, Axin, APC, and other proteins (not shown) binds and phosphorylates β-catenin, targeting it for ubiquitin-mediated proteosomal degradation. When Wnt binds the Frizzled receptor, Axin is engaged by the coreceptors LRP-5/6, Dishevelled (Dsh) is activated, and the destruction complex no longer phosphorylates β-catenin. β-catenin is released and enters the nucleus, where it activates target gene transcription together with TCF/LEF proteins. (A more complete diagram of this pathway can be found on the Wnt home page: http://www.stanford.edu/~rnusse/wntwindow.html). **E,** Notch signaling: In the absence of ligand binding, CSL transcription factors (*C*BF-1, *S*uppressor of Hairless, *L*AG-1) interact with a corepressor complex and inhibit the transcription of Notch target genes. The Notch receptor can be activated either by interaction with ligands (Delta, Delta-like, Serrate, Jagged) or the internalization of ligand into adjacent cells. Notch activation induces two cleavage events: TNFα converting enzyme (TACE) sheds the ectodomain, and γ-secretase releases the Notch intracellular domain (NICD) into the cytoplasm. NICD translocates to the nucleus, recruits a coactivator complex, and displaces the corepressor complex. The NICD complex then activates target gene transcription. (See color insert.)

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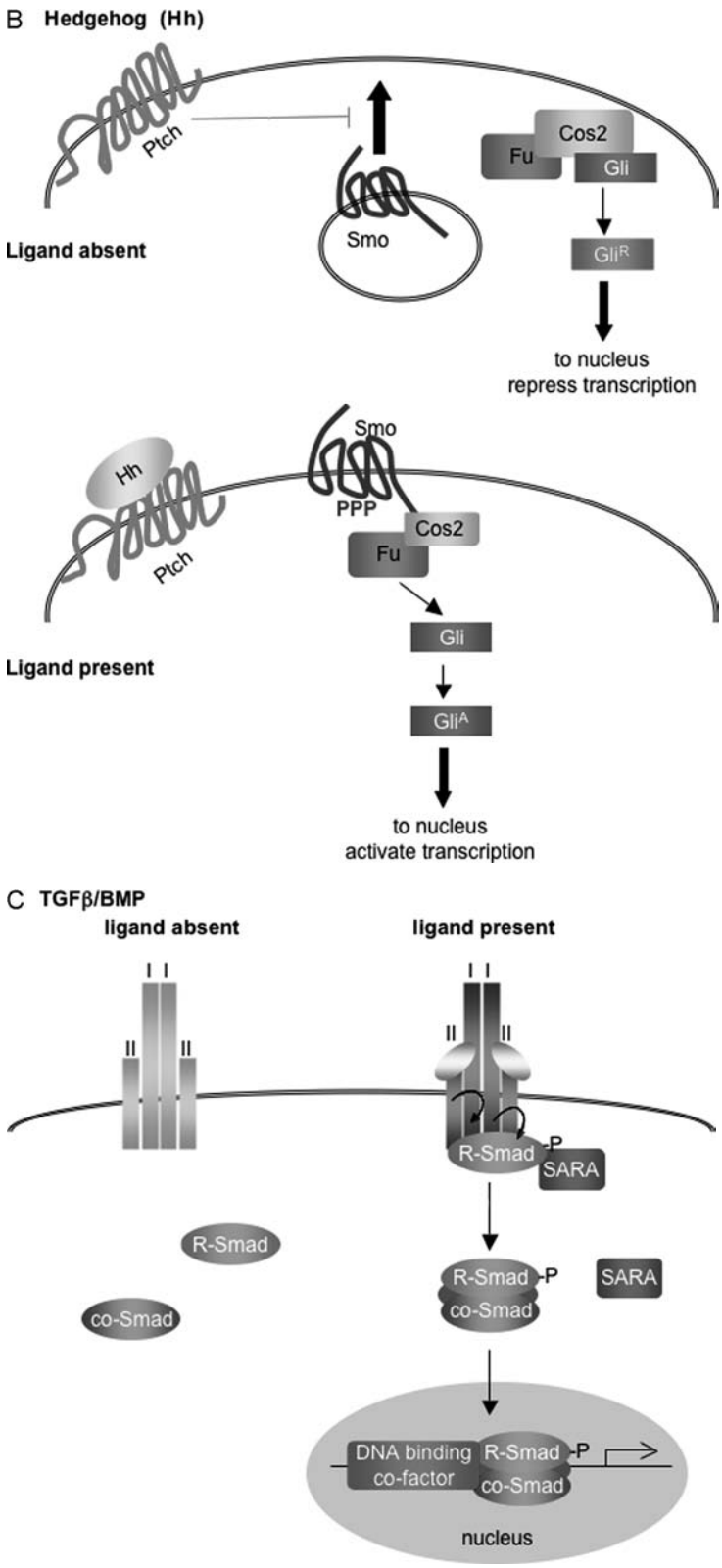
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Ptch
Smo
Cos2
Fu
Gli
GliR
to nucleus
repress transcription
Ligand present
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Ptch
Smo
PPP
Cos2
Fu
Gli
GliA
to nucleus
activate transcription
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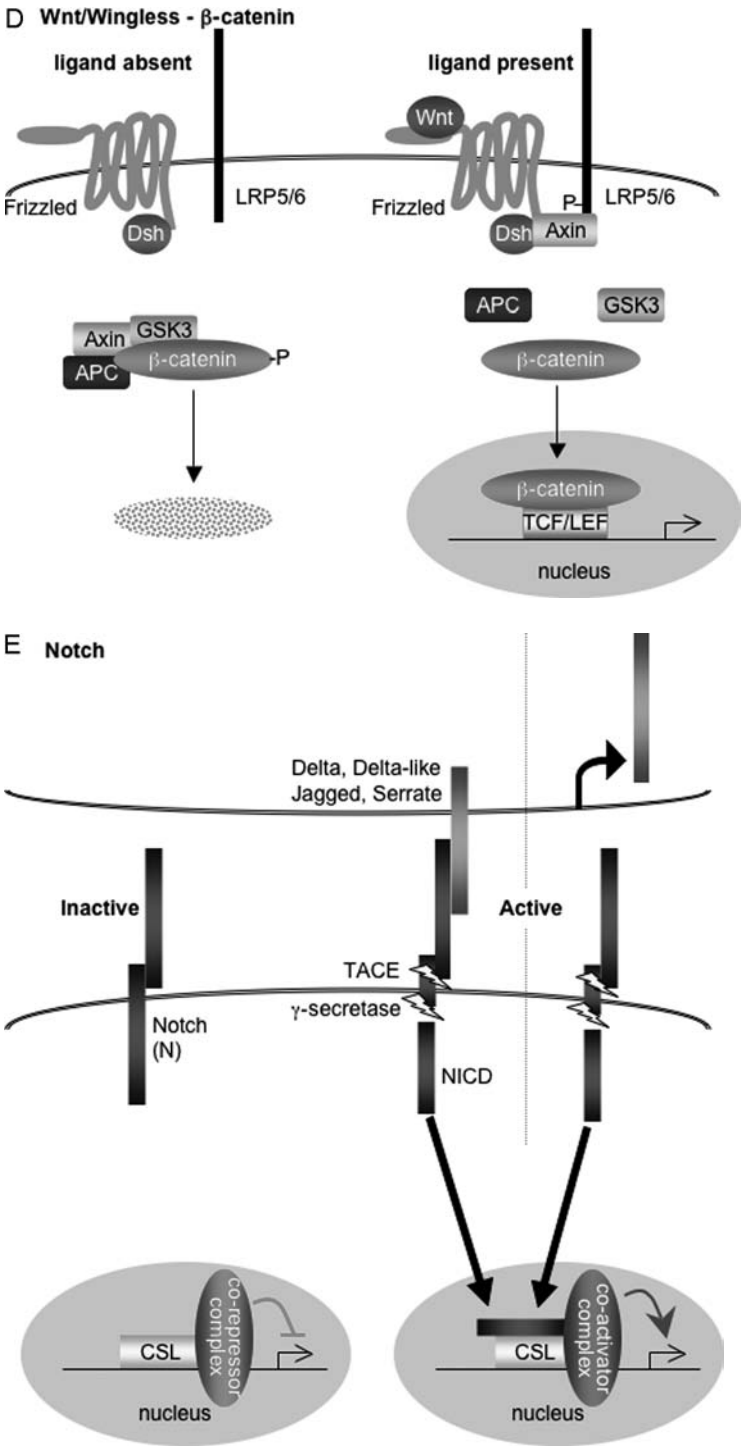
D Wnt/Wingless - β-catenin
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Dsh
Frizzled
P-
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Axin
Axin
GSK3
APC
β-catenin
-P
APC
GSK3
β-catenin
β-catenin
TCF/LEF
nucleus
E Notch
Delta, Delta-like
Jagged, Serrate
Inactive
Active
TACE
γ-secretase
Notch
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NICD
CSL
co-repressor
complex
nucleus
CSL
co-activator
complex
nucleus

Hedgehog (Hh), transforming growth factor β/bone morphogenetic proteins (TGFβ/BMP), Wnt/Wingless, and Notch (Figure 1.1) have been evolutionarily conserved and are used reiteratively during embryonic development to instruct cell behavior and fate and to coordinate tissue growth and patterning. Mutations that disrupt these pathways are associated with developmental and proliferative disorders that, in humans, include neurocristopathies and numerous forms of cancer.

In recent years, developmental signaling studies have begun to illuminate the mechanisms by which different pathways promote, antagonize, and/or synergize with one another in responding cells. These studies have been aided substantially by the development of increasingly sophisticated tools for genome-wide analysis and genetic manipulation. Genomic sequence data and high throughput assays have enabled classic genetic and biochemical screens to be performed and analyzed more efficiently. Importantly, by providing platforms for systematic genome-wide analysis, these technological advances have enabled screens to be less biased toward known genes and less dependent on specific phenotypic outcomes. However, genetic approaches remain essential complements to genomic studies because they are instrumental in addressing questions of mechanism and consequence (i.e., how specific proteins and interactions contribute to signaling and development).

In this chapter, we will discuss progress in four areas of developmental signaling: (1) the identification and characterization of novel signaling pathway components; (2) the distribution of ligand and the localization of signal transduction; (3) the mechanisms of signal transduction; and (4) the transcriptional targets of cell signaling events. As a result of space limitations, we are unable to provide a complete and comprehensive review of recent literature for any single signaling pathway (we refer interested readers to recent reviews for in-depth discussions of individual pathways: Schlessinger, 2000; Shi and Massagué, 2003; Kadesch, 2004; Lum and Beachy, 2004; Huangfu and Anderson, 2005b; Nusse, 2005). Instead, we focus on studies that illustrate novel conceptual advances and/or have used new approaches to address longstanding questions in the field.

I. IDENTIFICATION OF NOVEL SIGNALING PATHWAY COMPONENTS

A. Phenotype-Driven Screens *In Vivo*

Phenotype-driven screens, such as those used in early RTK studies, continue to prove invaluable for the identification of novel pathway components and modifiers in multiple developmental systems. The availability of annotated genome sequence data has made it possible to monitor the saturation of these screens, which are aided by collaborative efforts currently underway to mutate all coding genes in mouse, fly, and worm using various methods (see the databases and Web resources listed in Table 1.1). Screens in the different model systems have complemented one another and generated data sets that are overlapping but not identical. This may be in part because of the sensitivity of the phenotypes scored and the types of mutation (e.g., loss-of-function versus hypomorphic alleles) introduced in each system. However, studies of the Hh signaling pathway have suggested that some species-specific mechanisms are used to transduce cell–cell signals.

TABLE 1.1 Web-Based Resources for the Developmental Signaling Community

Web Site	Organisms	Contents
Model Organisms for Biomedical Research (NIH) http://www.nih.gov/science/models/	Fly, worm, mouse, fish, frog, and others	Links to Web resources for researchers, arranged by organism (including many sites listed below)
Database of Interacting Proteins http://dip.doe-mbi.ucla.edu/	Fly, worm, and mouse	Physical interactions
Enhancer Element Locator http://www.cs.helsinki.fi/u/kpalin/EEL/	Human, fish, mouse, rat, and dog	Enhancer elements (transcription factor binding sites) predicted on the basis of the comparative analysis of vertebrate genomes
FlyBase http://flybase.bio.indiana.edu/	Fly	Expression patterns, genetic interactions, gain- and loss-of-function mutation phenotypes, available alleles
PIMRider® (Hybrigenics' functional proteomic software) http://pim.hybrigenics.com/pimriderext/common/	Fly	Protein interaction mapping including genome-wide interactome data for *Drosophila* and a TGFβ-specific interactome data set
WormBase http://www.wormbase.org/	Worm	Developmental expression patterns, loss-of-function mutation phenotypes, available alleles
Worm Interactome http://vidal.dfci.harvard.edu/interactomedb/i-View/interactomeCurrent.pl	Worm	Protein–protein interaction database
Predictions of *C. elegans* Genetic Interactions (v 140) http://tenaya.caltech.edu:8000/ predict	Worm	Predicted functional interactions between *C. elegans* genes based on expression data, genetic and physical interactions in yeast, fly, and worm
International Gene Trap Consortium http://www.genetrap.org	Mouse	Available gene trap lines, expression data
Mouse ENU databases *The Sloan-Kettering Mouse Project* http://mouse.ski.mskcc.org/mutant/mutantBase.php *Baylor College of Medicine Mouse Genome Project: Mouse Mutagenesis for Development Defects* http://www.mouse-genome.bcm.tmc.edu/ENU/ENUMutantSources.asp	Mouse	Available mutants, phenotype data
GenePaint http://www.genepaint.org/Frameset.html	Mouse	Atlas of developmental expression patterns
	Mouse	

(Continued)

Web Site	Organisms	Contents
Edinburgh Mouse Atlas Project: EMAGE Gene Expression Database http://genex.hgu.mrc.ac.uk/Emage/database/ emageIntro.html		Atlas of developmental expression patterns
The Jackson Laboratory: Mouse Genome Informatics http://www.informatics.jax.org/menus/allsearch_menu.shtml	Mouse	Available mutant lines/alleles and phenotype data
The Zebrafish Information Network http://zfin.org/cgi-bin/webdriver?MIval=aa-ZDB_home.apg	Fish	Available mutant and transgenic lines, phenotype data, genetic maps, developmental gene expression patterns
Zebrafish Enhancer TRAP lines database (ZETRAP) (Parinov et al., 2004; Choo et al., 2006) http://plover.imcb.a-star.edu.sg/~zetrap/ZETRAP.htm	Fish	Available GFP lines (and patterns of GFP expression in each) generated by transposon-mediated enhancer trapping
ZF-MODELS: Zebrafish Models for Human Development and Disease http://www.zf-models.org/data/databases.html	Fish	Microarray (expression profiling) data, developmental expression patterns, loss-of-function phenotypes, available GFP lines

Key components of the Hh pathway are highly conserved (reviewed in Lum and Beachy, 2004; Huangfu and Anderson, 2005b). Hh signal transduction is controlled by the actions of the Patched (Ptc) receptor and the seven-pass transmembrane protein, Smoothened (Smo). In the absence of Hh, Ptc inhibits Smo from transducing signals. Hh binding to Ptc relieves this inhibition and enables Smo to activate a cytoplasmic signal transduction pathway that culminates in the proteolysis and nuclear translocation of an activating transcription factor (known as Gli in vertebrates and Ci in *Drosophila*). In mice, ENU mutagenesis screens identified cilia and intraflagellar transport proteins as essential components of the Hh pathway that act downstream of Ptc and Smo (Huangfu et al., 2003; Huangfu and Anderson, 2005a). Functional studies have demonstrated that activation of the Hh pathway in vertebrates induces the localization of Smo, Gli2 and Gli3, and other relevant proteins to cilia; a cilia localization motif on Smo is essential for normal Hh responses in cultured cells and zebrafish (Corbit et al., 2005; Haycraft et al., 2005). By contrast, intraflagellar transport mutations do not cause Hh-like phenotypes in *Drosophila*, and, in this organism, Hh-responsive cells do not have cilia (Ray et al., 1999; Han et al., 2003; Avidor-Reiss et al., 2004). *Drosophila* Smo accumulates at the plasma membrane upon Hh stimulation, whereas the vertebrate ortholog gets internalized. Furthermore, mammalian and fly Smo proteins are phosphorylated on different residues in response to Hh. Phosphorylation is required for the internalization of mammalian Smo

and for downstream signal transduction (Denef et al., 2000; Zhu et al., 2003; Chen et al., 2004a; Zhang et al., 2004; Apionishev et al., 2005). Likely as a result of such differences, antagonists of Hh signaling have disparate effects in flies and mice (Incardona et al., 1998; Taipale et al., 2000; Chen et al., 2002). These studies pose the challenge of discriminating evolutionarily conserved mechanisms from species-specific mechanisms of cell–cell signaling.

B. Systems Biology Approaches to the Identification of Signaling Pathway Components

Random, phenotype-driven mutagenesis screens are now being supplemented with sequence-driven genome-wide screens that do not rely on chance to reach saturation. These new approaches provide several advantages over classical techniques. Importantly, they are not reliant on phenotypic output, and so are capable of identifying genes that contribute to multiple cellular processes or pathways. These genes would likely have pleiotropic mutant phenotypes and therefore be discarded in screens for pathway-specific phenotypes. In addition, genome-wide screens can identify factors that have an impact on cell–cell signaling but are not essential for a normal developmental outcome (e.g., because of redundant or compensatory pathways).

Three types of genome-wide screens that have been used in signaling studies include *in vitro* RNA interference (RNAi) screens, protein interaction mapping (genome-wide yeast two hybrid [Y2H]), and developmental synexpression analysis. None of these approaches in isolation is sufficient to define signaling pathways and the requirements for individual components or interactions *in vivo*. However, each provides a platform for comprehensively scanning the genome and generating new models of cell–cell signaling.

1. RNAi Screens in Cultured Cells

RNAi uses short, double-stranded RNAs to trigger the degradation of target mRNAs species. This was developed as an experimental tool for work with *C. elegans,* in which it is now widely used for loss-of-function studies and phenotype-driven screens (Fire et al., 1998; Wang and Barr, 2005). Recently, genome-wide screens have been developed that use RNAi in *Drosophila* embryonic imaginal disc cell cultures (clone-8 cells) to identify novel signaling pathway components (Lum et al., 2003). In these screens, clone-8 cells are cotransfected with a pathway-responsive luciferase reporter and a comprehensive library of RNAi constructs. The products of known *Drosophila* coding genes are systematically tested for their ability to affect signaling pathway output as assayed by reporter activity.

The original clone-8 screens used Hh-responsive transcriptional reporters. RNAi of known Hh pathway genes altered luciferase activity in this system, validating the approach. In addition, numerous genes previously unassociated with Hh signaling were found to modify Hh reporter activity and to interact genetically with known Hh pathway members (Lum et al., 2003; Nybakken et al., 2005). Some of these genes belong to classes traditionally associated with cell–cell signaling; these include a heparan sulfate proteoglycan (Dally-like, which was previously implicated in Wnt signaling), a homeodomain gene, three kinases (CK1α, Pitslre1, and Cdk9), and a phosphatase (PP2A). Interestingly, the screens also indicated that the Hh pathway is affected by factors involved in more general cellular processes, including ribosome and proteosome function, RNA regulation and splicing, and vesicle

trafficking. Although the disruption of such genes would likely cause pleiotropic phenotypes *in vivo*, several lines of evidence suggest that they are bona fide components or modifiers of the Hh pathway. They were independently identified in two clone-8 RNAi screens, although not all genes required for splicing, transcription, and so on altered Hh reporter activity in these experiments. Furthermore, such genes have been identified (albeit at a low frequency) *in vivo* in screens that rely on hypomorphic alleles and/or clonal analysis (Eggenschwiler et al., 2001; Huangfu et al., 2003; Collins and Cohen, 2005; Huangfu and Anderson, 2005a). The results of clone-8 RNAi screens greatly expand the known landscape of Hh signaling. Further studies are now needed to determine how the novel Hh modifier genes fit into current models of the signaling pathway.

Similar RNAi screens with different transcriptional reporters have been used to scan the genome for genes that impact JAK/STAT and Wnt signaling. Like the Hh studies, these screens also identified proteins used in other signaling pathways as well as factors involved in general cellular processes (Baeg et al., 2005; DasGupta et al., 2005; Müller et al., 2005). Parallel screens in this system may prove useful for identifying points of crosstalk between pathways.

2. Interactome Mapping

Tewari et al. recently used Y2H assays in a genome-wide screen for *C. elegans* proteins that interact physically with known members of the TGFβ pathway (the basic pathway is diagrammed in Figure 1.1, C). They thus generated an "interactome" map describing physical interactions among 59 proteins, only four of which had previously been assigned to the TGFβ signaling pathway. Novel components of this biochemically defined interactome were then analyzed *in vivo* expression studies determined whether they are expressed in TGFβ-dependent contexts, and double RNAi experiments identified genetic interactions with previously known TGFβ pathway genes. Thus, several new proteins were modeled into the TGFβ signaling network, including filamin, the TTX-1 homeobox protein, Swi/Snf chromatin remodeling factors, and Hsp90 (Tewari et al., 2004). Additional biochemical and functional studies are needed to characterize the roles of the interactome components in TGFβ signal transduction and development.

An important feature of interactome mapping is that it is not hindered by compensatory mechanisms that may mask roles of pathway members in other assays. In addition, novel components identified using this approach can be directly modeled into known signal transduction pathways on the basis of physical and genetic interactions. Genome-wide Y2H analyses have now been reported for *C. elegans* and *Drosophila*, and protein–protein interaction data for multiple systems have been compiled into an interactive public database (Table 1.1, Database of interacting proteins; Xenarios et al., 2002; Giot et al., 2003; Li et al., 2004a; Formstecher et al., 2005). Thus, interactome mapping can now be done to some degree *in silico* as a starting point or modeling tool for signaling studies. The selection of different bait proteins in future Y2H screens will continue to enrich pathway-specific data sets. However, a challenge for future studies is to develop methods for mapping physical interactions in cell systems that are more representative of biological contexts. Phosphorylation events, which are known to figure prominently into signal transduction, are not recapitulated in yeast. Although phosphomimetic amino acids can be substituted into bait proteins

for Y2H studies, the results of these studies are limited to proteins that do not require phosphorylation for the assayed interaction. Furthermore, interactome components are likely to be cell type specific; comparative studies in different cell systems may illuminate context-specific mechanisms of signal transduction.

3. Identification of Synexpression Groups

Signaling networks have increased in complexity during evolution as a result of gene duplication events and the incorporation of redundant or compensatory signaling events. Many proteins in these networks have modular and conserved protein interaction domains (e.g., phosphotyrosine-binding domains, src homology domains) that are fairly promiscuous in biochemical assays. Furthermore, *in vivo* analyses have indicated that many pathways use context-specific mechanisms of signal transduction during development (discussed in section III). It has therefore become a significant challenge to determine which proteins are functionally associated in distinct biological contexts. Developmental synexpression analysis has proven useful for generating models of ligand-receptor relationships, signal transduction pathways, and regulatory events that comprise signaling modules *in vivo*.

Genome-wide expression screens performed predominantly in zebrafish led to the identification of an evolutionarily conserved Fgf8 synexpression group that contains several regulators of the RTK-Ras-MAPK pathway, namely Sprouty proteins, the transmembrane protein Synexpressed with FGF (Sef), and MAPK phosphatase 3 (Mkp3; Kudoh et al., 2001; Fürthauer et al., 2002; Tsang et al., 2002; Kawakami et al., 2003; Tsang et al., 2004). As might be expected for antagonists of a broadly used signal transduction cascade, these proteins do not exhibit strict RTK specificity in biochemical assays (Camps et al., 1998; Reich et al., 1999; Tsang et al., 2002; Kovalenko et al., 2003; Preger et al., 2004; Torii et al., 2004). However, synexpression suggests that they are required in Fgf8-expressing tissues, and functional studies have indicated that they antagonize Fgf signaling *in vivo* (Kramer et al., 1999; Fürthauer et al., 2002; Tsang et al., 2002; Kawakami et al., 2003). This does not preclude the possibility that they inhibit signaling by other RTKs at sites of Fgf8 expression. Indeed, in *Drosophila*, Sprouty and Mkp3 also regulate Egfr signals, and Mkp3 is expressed in contexts that are dependent on multiple RTKs (Kramer et al., 1999; Kim et al., 2004; Gómez et al., 2005). Genetic interaction studies are needed to determine the targets of Sef, Mkp3, and Sprouty regulation in vertebrates.

The functions of some signaling pathways are conserved across species: for example, the Fgf/Fgfr pathway is required for branching morphogenesis during lung and trachea development in mammals and flies, respectively (Reichman-Fried et al., 1994; Sutherland et al., 1996; Min et al., 1998; Sekine et al., 1999). Conservation of expression patterns across species is highly suggestive of functional conservation, and so expression profiling in different model organisms can help to identify gene functions. One member of the Fgf8 synexpression group in both planaria and vertebrates is the secreted Fgfr-like protein Isthmin (also known as nou-darake, Fgfr-Like 1). The roles of this protein in vertebrates have been elusive in loss-of-function studies, perhaps as a result of compensatory or redundant regulatory pathways (Cebria et al., 2002; Pera et al., 2002). However, loss of *isthmin/nou-darake* in planaria results in an expansion of anterior neural tissues during regeneration; this is suppressed by the simultaneous silencing of *Fgfr1* and *Fgfr2* (Pera et al., 2002).

These results implicate isthmin as an Fgf antagonist that restricts neural proliferation and/or fate, and they suggest that the vertebrate ortholog may have similar roles in restricting Fgf signals during neural stem cell and/or anterior central nervous system development.

The integration of synexpression data with biochemical and loss-of-function data could notably expedite future studies of developmental cell–cell signaling. Several Web-based resources that detail developmental expression patterns are currently available to the community (see Table 1.1). These data can provide clues as to the context-specific usage of signaling proteins and thus help to refine models of *in vivo* signal transduction.

II. DISTRIBUTION/LOCALIZATION OF LIGAND AND SIGNAL TRANSDUCTION

Many components and/or modifiers of signaling pathways function within cells or the extracellular space to ensure the proper localization of signals and their biochemical responses. Heparan sulfate proteoglycans (HSPGs) contribute significantly to this aspect of cell signaling by modulating the distribution and/or activity of Wnt, TGFβ/BMP, Fgf, and Hh proteins. A number of studies have addressed the roles of HSPG core proteins and synthesis or modifying enzymes in developmental cell signaling. In the mouse, an ENU-induced mutation in UDP-glucose dehydrogenase (Ugdh, a glycosaminoglycan synthesis factor) was found to cause recessive mesodermal phenotypes reminiscent of *Fgf8* and *Fgfr1* null embryos (García-García and Anderson, 2003). Similarly, mutations in Ugdh (*sugarless*) and other HSPG synthesis and processing enzymes disrupt Fgf-dependent development in *Drosophila* (Lin et al., 1999). Biochemical studies have demonstrated that heparan sulfate is essential for high-affinity Fgf–Fgfr binding and that Fgfs and Fgfrs have distinct affinities for different types of HSPGs (Ornitz, 2000; Mohammadi et al., 2005). Additional roles of heparan sulfates have been identified in *Drosophila* imaginal wing disc studies. In this context, HSPGs including Dally and Dally-like are required for long-range Hh signaling, cell surface accumulation and tissue distribution of Wnt and Hh, and stability or transport of Decapentaplegic (Dpp, a *Drosophila* BMP ortholog) as it travels across the wing disc epithelium (reviewed in Häcker et al., 2005).

Posttranscriptional and/or posttranslational modifications of ligands can also restrict movement within a tissue and thus enhance local signaling. For example, the diffusion of some mammalian Pdgf/Vegf ligands is regulated by alternative splicing of a "retention signal" motif, which is a C-terminal stretch of positively charged residues that can keep these ligands associated with producing cells (Eriksson and Alitalo, 1999; Heldin and Westermark, 1999). The Pdgfb retention motif is essential *in vivo* for its local actions: genetic ablation of the motif in $Pdgfb^{ret/ret}$mice leads to defects in pericyte number, vascular remodeling, and the association of Pdgfrβ-expressing pericytes with the Pdgfb-expressing vascular endothelium (Lindblom et al., 2003). However, the phenotypes of these mice are less severe than those of $Pdgfb^{-/-}$ and $Pdgfr\beta^{-/-}$ mice; this suggests that some roles of Pdgfb do not require local retention (Levéen et al., 1994; Soriano, 1994; Lindblom et al., 2003).

Intrinsic ligand structure and posttranslational modifications, such as lipid conjugation, tether some signaling proteins to cell membranes. Pathways leading to the synthesis, conjugation, and release/cleavage of membrane-associated moieties likely have an impact on the activities of these signals, which

include Notch ligands and ephrins. The ephrins are ligands that are associated with plasma membranes by GPI groups (A class) or transmembrane domains (B class). This restricts their activities to signaling between adjacent cells, and it enables them to function both in "forward" signaling to their cognate receptors as well as in "reverse" signaling in cells in which they are expressed (reviewed in Davy and Soriano, 2005; see Chapter 21). Genetic studies have demonstrated that reverse signaling via a PDZ interaction domain is essential for a subset of ephrin B1 roles during mouse embryogenesis (Davy et al., 2004). Membrane tethering of other types of ligand may similarly facilitate reverse signaling either directly or through associated proteins.

Mechanisms of localizing signaling proteins and their responses have been extensively studied in *Drosophila* imaginal wing discs, where secreted morphogens form gradients that induce different fates at different activity thresholds. Several models have been proposed to explain how secreted signaling molecules form gradients and reach target cells several cell diameters away from their sites of origin. According to one model, morphogens diffuse through the extracellular space; local concentration and activity are determined by factors that modulate ligand secretion, diffusion, stability, and receptor-mediated uptake. In the case of BMP signaling, ligand diffusion and stability are notably affected by auxiliary factors, including Short gastrulation (Sog), Twisted gastrulation (Tsg), and Tolloid (Tld; reviewed in O'Connor et al., 2006). Tsg facilitates Sog/Dpp binding in a trimeric complex that enables Sog, which is a Dpp antagonist, to keep the ligand inactive for extracellular transport across a tissue. At target sites, the protease Tld cleaves Sog, releasing Dpp to act locally. Combining mathematical modeling with experimental genetics, Mizutani et al. (2005) demonstrated that a diffusion model incorporating the effects of these proteins can recapitulate the BMP ligand gradient as well as the nonsynonymous BMP activity (phospho-Smad) gradient in *Drosophila* wing discs.

Although some secreted signaling proteins may be distributed by extracellular diffusion, imaging studies in *Drosophila* imaginal wing discs have suggested that more active mechanisms also contribute to signal localization. In unfixed wing discs expressing GFP, "cytonemes" (thin, actin-based membrane extensions that are several cell diameters long) extend from the apical surface of wing disc cells toward sites of either Dpp or Wg expression (Figure 1.2, A; Ramirez-Weber and Kornberg, 1999). Cytoneme formation in wing disc epithelia is Dpp-dependent, and the extensions are polarized toward Dpp or Wg only in regions where these factors act as morphogens. Interestingly, the Dpp receptor Thickveins (Tkv) is expressed on and moves directionally within Dpp-oriented cytonemes (Hsiung et al., 2005). Together, these data suggest that long-range actions of Dpp are mediated, at least in part, by the extension of receptor-expressing cytonemes toward sites of Dpp production. Similar structures may also guide chemotaxis in some contexts: actin-based cytonemes that contain Breathless (Btl, an Fgfr) extend toward sources of Branchless (Bnl, an Fgf) during the third instar larval migration of *Drosophila* tracheoblasts (Sato and Kornberg, 2002).

Imaging studies using a GFP-Dpp transgene led to a third model of Dpp localization in imaginal disc epithelia. Using this transgene, Teleman and Cohen directly visualized the ligand and found that it localizes to endocytic vesicles and is concentrated basally, whereas cytonemes protrude apically from wing disc cells. On the basis of these findings, the authors proposed that the Dpp gradient is formed via cycles of endocytosis and secretion that

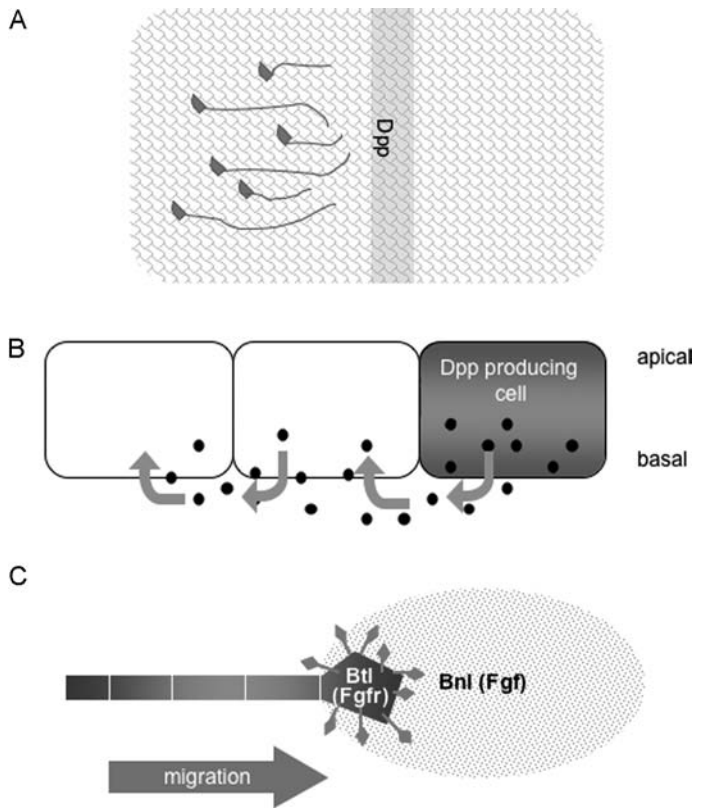

FIGURE 1.2 **Mechanisms of signaling protein transport observed in *Drosophila*. A,** Tkv, the receptor for Dpp, is expressed at the tips of cytonemes, which are long filamentous protrusions that extend from apical cell surfaces in imaginal wing discs toward the source of Dpp patterning signals (representative individual cells are diagrammed in green). **B,** Transcytosis (i.e., repeated cycles of endocytosis and secretion) moves Dpp across the wing disc epithelium. Dpp-containing vesicles are concentrated basally. **C,** Btl/Bnl signaling is required for tracheoblast migration. During branching morphogenesis, Btl induces the formation of short, cytoplasmic extensions on Bnl-expressing cells at the tips of tracheal branches. The filopodia-like structures observed in this context contain both actin and microtubules, and are not polarized toward ligand. (See color insert.)

transport Dpp within cells across the epithelial sheet (Figure 1.2, B; Teleman and Cohen, 2000). The cytoneme and GFP-Dpp studies may highlight distinct aspects of Dpp gradient formation. In support of this, ligand (Dpp) and response (phospho-Smad) gradients in the wing disc differ from one another, indicating that Dpp signaling activity is modulated at or downstream of the receptor (Teleman and Cohen, 2000).

Live imaging studies revealed that Bnl/Btl signaling induces another type of membrane extension in tracheal cells during branching morphogenesis. In this context, cells at the tips of tracheal branches extend numerous fine protrusions in response to Bnl (Figure 1.2, C). Unlike cytonemes, these filopodia-like structures contain both actin and microtubules, are relatively short, and are not polarized toward Bnl ligand (Ribeiro et al., 2002). It is not yet clear whether these structures are involved in Btl/Bnl signal transduction or localization.

Many aspects of developmental signaling are highly conserved, and so it is likely that the mechanisms of signal relay observed in *Drosophila* are used in other model systems. However, experiments in vertebrates have not yet

validated this hypothesis. In *Xenopus*, embryo cocultures were used to examine mechanisms of long-range signaling using a fluorescently tagged TGFβ ligand (Xnr2). This ligand induced transcriptional responses at a distance from secreting cells, but no cytoneme-like extensions were observed that were of sufficient length to explain the range of ligand action. Furthermore, Xnr2 was not observed in vesicles, and its transport did not rely on endocytosis. Thus, the authors concluded that Xnr2 is distributed in *Xenopus* embryos by diffusion rather than by cytonemes, filopodia, argosomes (vesicular structures), or transcytosis (Williams et al., 2004). This may reflect differences in the experimental systems, ligand- or context-specific mechanisms of signal relay, or the ability of fixation and imaging techniques to capture and preserve delicate membranous or vesicular structures. Further knowledge of the composition, formation, and mechanisms of action of ligand/receptor transport structures will greatly facilitate future studies in different model systems.

III. MECHANISMS OF SIGNAL TRANSDUCTION

Whereas loss-of-function alleles have revealed essential functions of many cell–cell signaling factors during development, more subtle and directed mutations are required to analyze signaling mechanisms. These mutations eliminate or alter specific protein–protein interactions and/or sites through which protein activity is regulated, and they are often designed after biochemical models. Recent *in vivo* studies using these types of alleles have begun to shed light on how functional specificity is achieved within protein families and how different signaling pathways intersect within a responding cell.

A. Specificity of Signal Transduction by Related Receptors

Two longstanding aims in the developmental signaling field have been to determine how closely related signals drive distinct responses *in vivo* and how individual receptors elicit context-specific responses over the course of development (discussed in Tan and Kim, 1999; Simon, 2000). Among related growth factor receptors, functional specificity could be achieved through differential utilization of and/or affinity for effector proteins; differences in the localization, duration, or amplitude of signal activation; or the context-specific availability of factors that modulate cellular responses.

Several lines of evidence have demonstrated that, despite biochemical similarities observed *in vitro*, members of the RTK superfamily drive non-equivalent signals *in vivo*. In *Drosophila*, the signaling domains of Torso and DER (*Drosophila* Egfr) drive migration responses to Btl activation incompletely and to different degrees in chimeric receptor rescue experiments (Dossenbach et al., 2001). A molecular explanation for this was suggested by the recent finding that, during tracheal branching morphogenesis, Btl and DER differ in their requirements for the downstream transcriptional effector Pointed (despite common activation of the Ras-MAPK pathway; Cabernard and Affolter, 2005). Similarly, chimeric receptor experiments performed in the mouse have shown that RTKs have distinct developmental potentials and transduce non-equivalent signals. The *Drosophila* Torso RTK signaling domain incompletely rescues Pdgfrα functions *in vivo* and activates only a subset of Pdgfrα-activated transduction pathways in primary cells (Hamilton et al., 2003). Likewise, the Pdgfrβ signaling domain is unable to drive Fgfr1

responses during embryonic development (Hoch, 2005). By contrast, the Fgfr1 signaling domain activates more potent signaling responses than Pdgfrα or Torso, and Pdgfrα/Fgfr1 chimeric receptor-expressing embryos exhibit dominant gain-of-function phenotypes (Hamilton et al., 2003).

Pdgfr and Vegfr studies have demonstrated that even RTKs within subfamilies transduce distinctive signals. The Pdgfrα signaling domain drives weaker MAPK responses than that of Pdgfrβ in cultured embryonic cells. In addition, the Pdgfrβ signaling domain can fully rescue Pdgfrα-dependent development *in vivo*, whereas the converse is not true (Klinghoffer et al., 2001). The differential recruitment of effector proteins may contribute to the disparity in Pdgfr signaling potential. Pdgfrα transduces signals predominantly via a single effector (PI3K) recruitment site during embryogenesis, despite its biochemical ability to engage proteins at additional sites (Klinghoffer et al., 2002). In contrast, multiple effector pathways contribute additively to Pdgfrβ functions in mice, as has also been reported for Torso in *Drosophila* (Gayko et al., 1999; Tallquist et al., 2003). The selective use of one pathway may limit the amplitude and variability of Pdgfrα responses, and may reflect the affinity or availability of effector proteins for this receptor.

Within the Vegfr subfamily of RTKs, Vegfr2 is thought to be the principal activator of signal transduction. This isoform responds to ligand with heightened receptor kinase and MAPK activities as compared with Vegfr1. These two receptors are coexpressed *in vivo*, and chimeric receptor studies have shown that Vegfr1 serves to regulate the activity of Vegfr2. Interestingly, different Vegfr1 ligands specify distinct modes of Vegfr2 regulation (inhibition versus potentiation; Rahimi et al., 2000; Autiero et al., 2003; Meyer and Rahimi, 2003; Roberts et al., 2004). The functional specialization of Vegfrs has been attributed to an amino acid change in the activation loop of Vegfr1 at a residue that is highly conserved among other class III RTKs (Meyer et al., 2006). Within several RTK subfamilies, homo- and heterodimers can form *in vitro*, but the significance of this observation *in vivo* and the consequences for downstream signaling are not known. The Vegfr findings introduce the possibility that subunits within other heterodimers have distinct functions that differentiate the signals transduced by homo- versus heterodimers.

Fgfr1 and Fgfr2 have been shown to signal through adaptors (Frs2,3) that are distinctive among RTK effectors in that they interact constitutively with these receptors instead of being recruited after ligand-dependent activation (Wang et al., 1996; Kouhara et al., 1997; Xu et al., 1998). Biochemical studies implicated Frs adaptors in MAPK and PI3K signaling downstream of Fgfrs (Wang et al., 1996; Xu et al., 1998; Hadari et al., 2001). However, the Fgfr1–Frs interaction is required only for a subset of Fgfr1 functions during mouse embryogenesis (Hoch and Soriano, 2006). Furthermore, in primary embryonic cells, this signaling event affects basal Fgfr2 activity but is not essential for MAPK activation responses to Fgf (Hoch and Soriano, 2006). Recently, Frs adaptors have been implicated in crosstalk and feedback regulation among Fgfrs and other RTKs. Activated Frs2 can recruit Cbl and instigate the ubiquitin-mediated degradation of Frs2 and Fgfrs (Wong et al., 2002). Frs2 is also threonine phosphorylated in response to Fgfs and other RTK-mediated signals; this inhibits Frs-mediated signaling to the MAPK and PI3K pathways (Lax et al., 2002). Finally, SHP2 and Src, which can both be activated downstream of Frs2, modulate the tyrosine phosphorylation of Sprouty proteins, which could impact signaling by several RTKs (Hanafusa et al., 2002; Fong et al., 2003; Hanafusa et al., 2004;

Li et al., 2004b; Jarvis et al., 2006). Further studies are needed to assess the contribution of these regulatory events to Frs functions *in vivo*. The uniquely constitutive association of Frs adaptors with Fgfr1 and Fgfr2 may confer preferential regulation of or sensitivity to Frs-mediated feedback regulation.

B. Crosstalk Between Signaling Pathways Occurring in the Cytoplasm

We are only beginning to understand the molecular mechanisms by which signaling pathways interact, although crosstalk has long been suggested by the results of tissue explant and recombination experiments. One mechanism of crosstalk that has been identified in BMP/TGFβ studies involves the combinatorial control of pathway intermediates. BMP/TGFβ family members signal through a small number of receptors that phosphorylate C termini of Smad proteins, thus activating these effectors to form trimers, translocate into the nucleus, and regulate transcription (Figure 1.1, C). MAPK (Erk, Jnk, p38) antagonizes this pathway by phosphorylating Smads at residues in their linker domains (Figure 1.3, A). This inhibits Smad nuclear translocation thereby blocking transcriptional responses to BMP/TGFβ, and can also target Smads for ubiquitin-mediated degradation (reviewed in Massagué, 2003; Sapkota et al., 2007). BMP and TGFβ signals can also induce phosphorylation of the Smad linker domain, but this event is delayed compared to the C terminal phosphorylation and does not disrupt nuclear signaling (Sapkota et al., 2007). The roles of phosphatases in cell signaling are generally understudied as compared with kinases, but these two classes of enzymes are of equal importance in the regulation of signal transduction pathways. An RNAi screen in *Drosophila* S2 cells identified pyruvate dehydrogenase phosphatase as a phosphatase for BMP/TGFβ-responsive sites on the fly Smad ortholog (MAD) and the mammalian Smad1 (Chen et al., 2006). Similar screens could conceivably identify additional Smad kinases and phosphatases through which other pathways impinge on Smad phosphorylation and localization. The combinatorial control of Smads may enable the relatively simple BMP/TGFβ pathway to drive different cellular responses depending on the availability of other signals.

Recent *Xenopus* studies suggest that the MAPK-mediated antagonism of BMP signaling underlies neural fate induction in the early vertebrate embryo (Pera et al., 2003; Kuroda et al., 2005). Similarly, this crosstalk may redirect TGFβ/BMP responses in other developmental contexts receiving concomitant RTK-mediated signals. For example, Fgf signaling and BMP antagonism have been implicated in neural crest induction (LaBonne and Bronner-Fraser, 1998; Steventon et al., 2005; Wawersik et al., 2005). Additionally, in the limb bud, p38MAPK signaling is essential for some responses to BMP (Zuzarte-Luis et al., 2004). An analysis of Smad1 phosphorylation site mutants revealed that MAPK-responsive residues on the Smad1 linker domain are essential only in select contexts in mice; these include the development of the reproductive tract and germ cells and postnatal digestive tract homeostasis. By contrast, C terminal residues phosphorylated downstream of BMP signals are required broadly for Smad1-dependent development (Aubin et al., 2004). The discrepancy between the *Xenopus* and mouse results may reflect species–specific roles of Smad phosphorylation, or, alternatively, may be caused by the activities of other Smad isoforms in the two experimental systems. Future studies are needed to distinguish between these models and to determine the developmental requirements for crosstalk mediated by other Smad proteins.

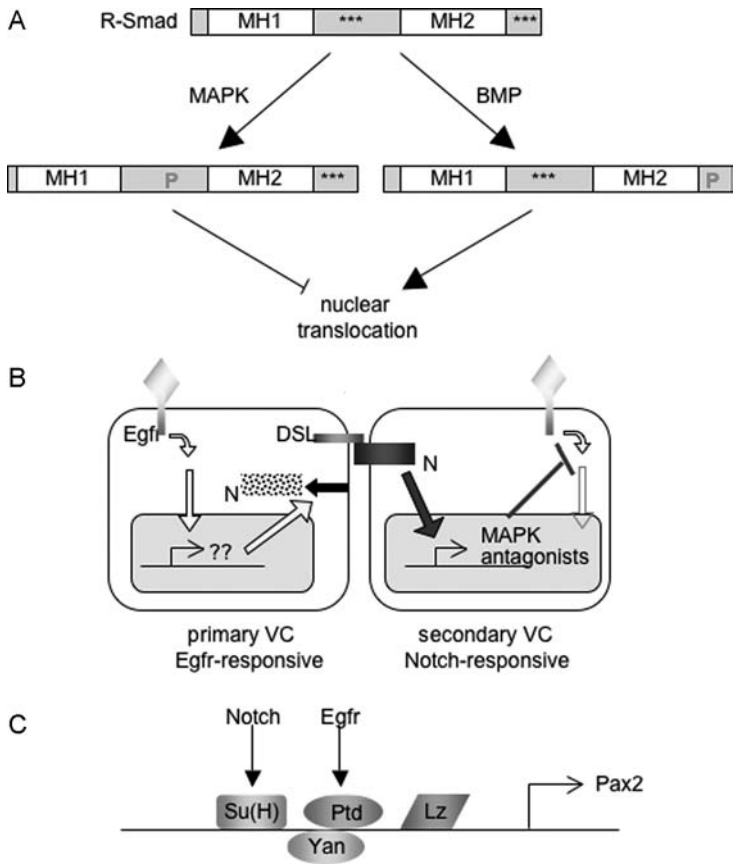

FIGURE 1.3 **Mechanisms of crosstalk between signaling pathways. A,** Convergence of pathways on common intermediates: BMP/TGFβ signaling leads to the C-terminal phosphorylation of Smad proteins, thereby promoting their trimerization and nuclear translocation. MAPK can phosphorylate serine residues in linker regions between the Mad homology domains (MH1, MH2) . This inhibits nuclear translocation of Smads and thus blocks transcriptional responses to BMP signaling. **B,** Transcriptional induction of proteins that modify cell signaling: In *C. elegans* vulval precursor cells (VC), antagonistic interactions between the Notch and Egfr pathways cause the primary and secondary VCs to be differentially responsive to these pathways. Egfr signaling (yellow) in the presumptive primary VC induces the transcription-dependent internalization and degradation of Notch, thus activating lateral inhibition signaling by Notch ligands (Delta, Serrate, LAG-1 [DSL]). This activates the Notch pathway in the secondary VC, which culminates in transcription of MAPK antagonists that block Egfr signal transduction. **C,** Combinatorial control of transcription: In *Drosophila* eye cone cells, expression of *Pax2* requires transcription factors activated by Notch (Suppressor of Hairless [Su(H)]) and Egfr signaling (Pointed [Ptd], Yan) as well as a regional transcription factor, Lozenge (Lz). Each of these transcription factors binds a distinct site in a *Pax2* enhancer element.

IV. TRANSCRIPTIONAL TARGETS OF SIGNALING PATHWAYS

A. Crosstalk Between Pathways Mediated by Transcriptional Regulation

Multiple mechanisms of crosstalk have been identified that involve transcriptional regulation. Transcriptional profiling studies have indicated that cell–cell signaling events commonly induce the expression of signaling and regulatory proteins that alter the responding cell's interactions with its environment. This

form of feedback regulation has been shown to modulate signaling both within and across pathways. During *C. elegans* vulva induction, Egfr and Notch signaling induce the transcription and/or activation of factors that establish reciprocal responsiveness to these pathways in neighboring cells (Figure 1.3, *B*). Egfr activation induces the internalization and degradation of Notch in the primary vulva cell. This transcription-dependent event enables Notch ligands to activate the receptor on neighboring cells and thus initiates lateral inhibition signaling (Shaye and Greenwald, 2002, 2005). Then, in secondary vulva cell precursors, Notch signaling induces the transcription of several MAPK pathway antagonists, thus inhibiting Egfr–Ras–MAPK signal transduction (Yoo et al., 2004).

In *Drosophila* eye studies, two additional mechanisms of crosstalk between Egfr and Notch signaling have been elucidated. In this context, proteins from the two pathways converge to coordinately regulate target gene expression. In one set of studies, Groucho, a transcriptional corepressor that acts downstream of Notch (and Wnt), was found to be a point of crosstalk with the Egfr–MAPK pathway: MAPK can phosphorylate Groucho and thus weaken its corepressor activity. In this way, Egfr signaling can derepress the transcription of Notch target genes. These results provided a mechanistic model to explain the previous observation that Groucho interacts genetically with both Notch and Egfr (Price et al., 1997; Hasson et al., 2005).

In cone cells of *Drosophila* eyes, transcription factors activated by Notch and Egfr signaling converge on an enhancer element to regulate *Pax2* expression. A regional transcription factor, Lozenge, also binds this enhancer, and the transcriptional response requires the occupancy of all three sites (i.e., coregulation by factors activated by the Notch and Egfr pathways as well as the context-specific factor; Figure 1.3, C). *Prospero* is also coordinately but distinctly regulated by transcription factors downstream of the Egfr and Sevenless RTK pathways in *Drosophila* (Xu et al., 2000). There is evidence that this mode of crosstalk has been conserved in vertebrates: the *Sox2* and *Cdx3* genes in *Xenopus* are coordinately regulated by Wnt and Fgf signals (Haremaki et al., 2003; Takemoto et al., 2006).

Recently, Hallikas et al. (2006) devised a computational tool to identify transcription factor binding sites, and they used it to scan the vertebrate genome *in silico* for targets of RTK, Hh, and Wnt signaling. Several putative targets were identified and validated through subsequent expression studies and cross-referencing with published work. Interestingly, this analysis indicated that there is significant overlap in the targets of Tcf (Wnt) and Gli (Hh) transcriptional regulation. The combinatorial control of enhancers may thus be a common means of crosstalk between these two pathways. These and other results were compiled in a searchable database of predicted enhancer elements for vertebrate genes (Enhancer Element Locator in Table 1.1).

B. Transcriptional Profiling of Signaling Responses

Many studies have characterized the transcriptional responses to signaling events since array technology was developed. For example, Fambrough et al. (1999) addressed the question of signaling specificity by comparing the transcriptional responses downstream of RTKs in cultured cells. Kit, Pdgfrβ and Fgfr1 were found to induce the transcription of the same set of genes in this system (with some quantitative differences), whereas Egfr

induced transcriptional responses that differed both qualitatively and quantitatively from these other RTKs. The disruption of effector binding sites on Pdgfrβ did not significantly affect its transcriptional response in these experiments, consistent with what was subsequently observed *in vivo* (Tallquist et al., 2003).

Biologically relevant transcriptional profiling relies on the selection of informative tissue or cell samples. In a recent screen for Wnt target genes, comparative expression analysis was performed using gastrulation-stage wild-type and *β-catenin* mutant mouse embryos. In addition to known Wnt target genes, several novel targets were identified in this study, including components of other signaling pathways (e.g., *Notch*) and genes expressed in domains of Wnt reporter activity during gastrulation. Some target genes (*Grsf1, Fragilis*) were further validated as Wnt-associated genes *in vivo:* embryos derived from RNAi knockdown embryonic stem cells recapitulated aspects of Wnt mutant phenotypes (Lickert et al., 2005).

In whole-embryo analyses, it is difficult to discern direct targets of signaling pathways from transcriptional changes that are secondary to developmental aberrations. Furthermore, different cell types and developmental contexts may respond to signals with distinct responses. For these reasons, profiling experiments would ideally use homogeneous cell populations that have not been immortalized or otherwise modified from their native state. High-fidelity cDNA amplification techniques are being developed to enable the profiling of single cells and small cell populations. This and similar technical advances will enable researchers to identify the transcriptional targets of signaling events in spatially or temporally restricted niches within developing embryos.

The results of profiling studies need to be substantiated in functional assays that demonstrate the significance of identified targets in mediating relevant cellular responses. To facilitate the transition from expression analysis to functional validation, Chen et al. (2004b) generated a microarray of cDNAs representing genes that were randomly mutated by retroviral gene trapping in ES cells. This chip—or gene trap array—can be used to profile transcriptional changes in wild-type versus mutant cells/tissues, uninduced versus induced cells, or cells at different stages of differentiation. Mutant mice can then be generated from archived mutant ES cells for the analysis of putative target genes *in vivo*. In an initial study, the gene trap array was used to assess transcriptional responses of mouse embryonic cells to Pdgfrα versus Pdgfrβ stimulation (Chen et al., 2004b). The functions of several novel Pdgf target genes identified, and their genetic interactions with Pdgfrs were then addressed *in vivo*. Results of these studies implicated Pdgfs in the modulation of signaling by other secreted molecules (e.g., sphingosine) identified the transcriptional targets required for specific aspects of Pdgf-dependent development, and suggested novel postnatal roles of Pdgf signaling (Schmahl et al., 2007).

V. CHALLENGES FOR FUTURE STUDIES OF DEVELOPMENTAL SIGNALING

We have highlighted four major areas of developmental signaling in which recent advances have been made using a combination of genetic and genomic tools. First, we discussed approaches that have been used to generate a global overview of factors that impact specific cell–cell signaling pathways. Next, we discussed the mechanisms underlying distinct steps in a signaling event, progressing from the secretion and transport of the signal to the signal

transduction events initiated by ligand-receptor binding. Finally, we discussed studies that address the outcome of cellular responses to environmental signals by examining transcriptional responses. Many key responses to cell–cell signaling (e.g., cell migration, adhesion, and cell cycle progression) may not require transcription; high-throughput assays need to be developed with which the molecular events underlying these responses can be explored.

One major challenge for future studies is to further elucidate the mechanisms of cell–cell signaling in developing organisms. An important result that has emerged from recent *in vivo* work is that different mechanisms are used to transmit and transduce signals in different cellular and developmental contexts. Results are not always transferable between systems as defined by organism, tissue, or cell type. Therefore, future studies of signaling mechanisms will require the use of genetically mosaic embryos and inducible alleles that are activated in a restricted manner by heat, irradiation, or locally expressed recombinases. To date, many such studies have used loss- or gain-of-function alleles, but more directed alleles are needed to isolate the roles of promiscuous signaling proteins.

Analyses of signaling pathways in isolation are instrumental for the elucidation of core pathway components and prominent signal transduction mechanisms. However, cells *in vivo* are commonly exposed to multiple concomitant cues. Thus, to fully understand cell–cell signaling, we need to transition from thinking of individual signaling pathways to considering how they are interwoven to form comprehensive signaling networks within responding cells. Recent advances in different areas of developmental signaling (particularly those that incorporate systems biology approaches) have begun to illuminate some mechanisms of crosstalk between major pathways. These include the convergence of signal transduction onto shared intermediates, transcriptional feedback loops, and the combinatorial regulation of transcription. Integration, convergence, synergy, or antagonism between signaling pathways can dramatically affect a cell's interactions with its environment. In addition, these and other mechanisms may confer preferential responsiveness to a particular signal or enable a cell to respond differently to distinct combinations of signals.

As new tools are developed for addressing developmental questions at a systems biology level, the amount of data generated in the field is growing exponentially. Consequently, biologists are growing increasingly reliant on computer scientists and computational biologists for data analysis, management, and access. Results from many experiments can no longer be contained within a standard journal article and instead require Web-based data supplements. Many collaborative efforts have been undertaken to centralize vast amounts of data in public databases and Web sites. However, several of these resources remain underused, largely as a result of insufficient publicity and a lack of infrastructure linking related data sets. Within the mouse community, this is especially apparent. Whereas the fly, worm, and fish communities have developed Web sites that comprehensively include expression, phenotype, genetic, and physical interaction data as well as available alleles and publication links, the mouse data sets are currently dispersed in several unlinked Web sites. It will take an enormous effort to integrate the information contained in these sites, but such an undertaking would create a tremendously valuable resource for the scientific community. A comprehensive mammalian database incorporating multiple types of mouse data as well as human genetic and phenotypic data could bridge developmental and medical research and make the networking of Web sites for different model organisms a far more accessible goal.

A wealth of information is contained within current Web-based resources, but the full significance of this data lies waiting to be unveiled in computational analyses that integrate different types of data. The power of this approach was demonstrated recently by Zhong and Sternberg (2006), who generated genome-wide predictions of functional interactions in *C. elegans* by integrating expression, phenotype, and physical and genetic interaction data from multiple model systems. Computational and experimental systems biology approaches provide exciting and essential complements to genetic and biochemical investigations of cell–cell signaling. Together, these different types of studies will elevate our understanding of developmental signaling to a new level in coming years.

SUMMARY

- Several cell–cell signaling pathways are used reiteratively to instruct developmental processes, and form complex networks within cells that we are only beginning to understand thanks to convergent genetic, genomic, and biochemical studies.
- Components and modifiers of developmental signaling pathways have been identified in several types of screens. These screens have revealed that specific pathways are affected by proteins involved in general cellular processes as well as factors that belong to more traditional signal transduction classes. Now, the challenge is to understand how newly implicated factors affect signaling and development *in vivo*.
- Signal (and signal transduction) localization is highly regulated *in vivo* by a variety of mechanisms, including regulated stability and/or diffusion, facilitated transport, the protrusion of cytoplasmic filaments containing receptors, and cycles of endocytosis and secretion.
- Recent studies using directed signaling alleles have identified molecular mechanisms by which closely related proteins drive distinct responses, and have shown that context-specific signaling mechanisms are used *in vivo*.
- Several mechanisms of antagonistic and synergistic crosstalk between pathways have been identified, including the coregulation of signaling intermediates, transcriptional feedback regulation, and the convergence of transcriptional effectors at target enhancer or promoter elements.
- Expression profiling studies to characterize the transcriptional responses to specific signaling events are constantly being improved by the use of increasingly relevant sample sources as well as amplification techniques. However, technologies still need to be developed that enable researchers to study other responses such as cell migration and proliferation in large-scale experiments.

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GLOSSARY OF TERMS

Argosome
A type of vesicle that is derived from the basolateral membranes in the *Drosophila* wing disc epithelium and transports a signaling protein across a field of cells; proposed to traverse the wing disc by repeated cycles of transcytosis.

Cell-autonomous
Affecting only the cell of origin.

Cell-nonautonomous
Having effects that are not restricted to the cell of origin, as does a secreted protein.

Cytoneme
A thin, actin-based cellular protrusion several cell diameters long that extends from the apical surface of a cell toward a source of signaling protein (ligand); first observed in *Drosophila* wing disc cells extending toward Dpp and Wg.

Dominant phenotype
A phenotype that results when a single mutant copy of a given gene functionally dominates over the second wild-type allele.

Effector protein/pathway
A signaling protein or pathway that drives a biochemical or cellular response to a stimulus or signaling event; for example, in RTK signaling, a protein or pathway that is activated in response to receptor activation through the recruitment of an adaptor or another protein to the active receptor.

Enhancer element
A region of DNA that affects gene transcription in *cis* through the recruitment of transcription factors or other DNA binding/modifying proteins.

ENU
N-ethyl N-nitrosourea; a chemical mutagen that induces point mutations in DNA in a dosage-dependent manner.

Epistasis
A functional interaction between nonallelic genes; the ability of one allele to suppress the phenotypic consequences of a second mutation, which typically indicates that the epistatic mutation is dominant or is downstream in a common genetic pathway.

Feedback regulation
A mechanism by which a signaling pathway regulates its own activity; for example, by activating a regulatory factor that alters signal transduction, by altering the sensitivity of the pathway to upstream signals, and/or by modifying the activity or interactions of proteins in the pathway.

Filopodia
Thin, short cellular protrusions that contain both actin and microtubules and are not polarized toward a source of signaling protein.

Gain-of-function mutation
A mutation that results in a hyperactive gene product due to deregulated expression or function; for example, a mutation that renders the gene product resistant to the effects of regulatory enzymes.

Genetic interaction
Functional synergy between two mutations that is suggestive of the gene products acting together in a given process or pathway. A genetic interaction is manifested through a compound mutant phenotype that is more pronounced than the sum of the two single mutant phenotypes; for example, disrupted development in an animal that is heterozygous for two mutations for which either heterozygous mutation (in isolation) does not result in a developmental phenotype.

Glycosyl phosphatidylinositol (GPI)
A type of phospholipid that is often conjugated to proteins and used to tether them to the plasma membrane.

Heparan sulfate proteoglycan (HSPG)
A macromolecule comprised of a core protein and glycosaminoglycan side chains of the heparan sulfate (polysaccharide) family; HSPGs are abundant in the extracellular matrix and are sometimes associated with plasma membranes via lipid moieties. They are important for many signaling events as revealed by the effects of mutations in HSPG core proteins and synthesis enzymes (e.g., those involved in appending the HS side groups).

Hypomorphic allele/mutation
A mutation that incompletely disrupts gene function and causes a phenotype that is less severe than a null (complete loss-of-function) mutation.

In silico
Computational, using informatics and computer-based resources.

Interactome
A large-scale protein interaction map, based on the results of biochemical assays testing all known coding gene products for their ability to interact physically with one or more protein(s) of interest.

Kinase
An enzyme that transfers a phosphate group to a substrate protein in an adenosine-triphosphate (ATP)–dependent reaction; often used in signal transduction to alter the activity or binding properties of a protein in a cascade.

Lateral inhibition
A signaling-mediated process by which one cell restricts the developmental potential or fate of its neighbor.

Loss-of-function mutation
An inactivating mutation that blocks gene expression or impairs the function of a gene product.

Morphogen
A protein that acts on target cells at a distance from its cell of origin, that forms an expression or activity gradient over a field of responsive cells, and that drives different cellular responses at different concentrations or activity thresholds.

Niche
A specific milieu defined temporally, spatially, and in some cases functionally; often during development, a given niche (e.g., a stem-cell niche) possesses

specialized characteristics as a result of the cellular composition of the niche itself as well as its interactions with nearby cells or proteins.

Phosphatase
An enzyme that removes a phosphate group from a substrate protein; like kinases, phosphatases are often used to regulate signal transduction.

Physical interaction
A direct binding interaction between two proteins.

Posttranscriptional modification
The modification of an mRNA after gene transcription but before translation into protein; for example, a splicing event.

Posttranslational modification
The modification of a protein; for example, phosphorylation, lipid conjugation, or cleavage.

Recessive phenotype
A phenotype that results from a mutation whose consequences can be functionally suppressed by a single wild-type allele; a mutation that only disrupts normal gene function in the homozygous state.

RNA interference (RNAi)
A means of knocking down the levels of one or more transcripts by introducing double-stranded or short-interfering RNAs to a cell and thus inducing the degradation of sequence-homologous mRNA.

Screen, expression
A screen based on development gene expression patterns.

Screen, phenotype-driven
A screen for proteins that affect the same developmental processes as assessed by developmental outcome, often performed using random mutagenesis approaches.

Screen, sequence-driven
A screen that uses genome sequence and annotation information together with gene-directed approaches to scan an entire genome for genes of interest.

Synexpression
Developmental coexpression.

Systems biology
The use of unbiased, high-throughput methods to simultaneously analyze all components of a biological system, thus providing a description of the whole system rather than its isolated components; for example, analyzing genome-wide changes in transcript or protein levels.

Transcriptional profiling
The analysis of mRNA expression, often using microarrays; in cell-signaling studies, comparative transcriptional profiling is often used to assess the transcriptional targets of a signaling pathway.

Transcriptional reporter
An experimental tool used to monitor the activity of a gene promoter or enhancer element; a protein of measurable activity or intensity (e.g.,

luciferase, β-galactosidase) driven by the transcriptional control elements of a gene of interest.

Transcytosis
The internalization, vesicular transport, and exocytosis of a secreted signaling protein that moves it through a cell and releases it into the extracellular space distal to its site of origin.

Yeast two hybrid (Y2H)
An experimental method used to assay for direct protein–protein interactions; with this method, a "bait" protein is fused to the DNA-binding domain of a transcription factor (TF), and a series of "fish" proteins are fused to the activation domain of the TF. When the bait and fish proteins physically interact, the proximity of the two TF domains render the complex capable of driving the expression of a reporter gene.

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2

FINDING GENE EXPRESSION CHANGES USING MICROARRAY TECHNOLOGY

TADAYOSHI HAYATA[1] and KEN W. Y. CHO[2]

[1]*Department of Molecular Pharmacology, Medical Research Institute, Tokyo Medical and Dental University, Tokyo, Japan*

[2]*Department of Developmental and Cell Biology, University of California, Irvine, CA*

INTRODUCTION

Fertilized eggs give rise to many millions of cells that represent hundreds of different cell types and that eventually form the complex structures of the adult. Each dividing cell makes numerous specific decisions about which genes to express among the tens of thousands of genes in its genome. If this tightly regulated process goes awry, an embryo may develop abnormalities or subsequently develop diseases as an adult. The regulatory programs that turn specific genes on or off are embedded in the genome of the organism. Therefore, a current major effort in biology is to understand the mechanisms by which gene networks are coordinated, thereby specifying paths of cellular differentiation. DNA microarray approaches provide biologists with the opportunity to generate expression inventories of all genes used to create the embryo. This information will lead to an improved understanding of how tissues and organs develop, which has clear implications for biomedicine.

Until recently, the task of cataloging the expression of thousands of genes to understand how they control animal development was an unrealistic goal for biologists. However, advances in genome sequencing and the development of high-throughput microarray approaches are moving this formidable task into the realm of reality. Although it is still challenging, scientists have begun to organize the expression of numerous genes into various groups sharing similar expression patterns. This holistic, genome-level view that involves the examination of the simultaneous readouts of all of the components is beginning to transform the way we view biological processes. Data from such studies has enabled researchers to link previously unsuspected genes to particular developmental

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pathways, mutations, and diseases. We will discuss the basic theory behind microarrays and how the technology is useful for biomedical research.

I. MICROARRAY PRINCIPLES

A. Basic Theory Behind Microarray Technology

The principle underlying the ability of microarrays to detect specific transcripts is solid support nucleic acid hybridization as first developed during the 1970s by Ed Southern for the classical Southern blot hybridization. Microarray chips are distant cousins of Southern blots in the sense that a microarray can be seen as an orderly arrangement of many miniaturized DNA "dot blots." Unlike standard dots blots, each dot of immobilized target DNA represents a single DNA species rather than a complex mixture of genomic DNA. Modern microarrays use DNA-bound platforms (slides or quartz wafers) containing very large numbers of genes arranged in an array distributed over a very small area (mm to cm) (Schena et al., 1995; Lockhart et al., 1996). This miniaturization makes it possible to examine the expression of large numbers of genes in a small sample size. Therefore, scientists are able to address increasingly complex questions and to perform intricate experiments to address gene expression profiling, genotyping, and the global effects of gain- and loss-of-function for specific genes.

Several methods are used to fabricate microarrays (Schena, 2000). One popular method, known as spotted (glass) arrays, uses DNA generated from polymerase chain reaction (PCR) amplicons (size range, 0.5 kb to 2 kb) or long oligonucleotides (size range, 50 bp to 70 bp) mechanically deposited on the surface of chemically coated glass substrates (Figure 2.1). Oligonucleotide arrays are designed to match specific subsequences in known or predicted mRNAs. Both types of arrays use a robotically driven device containing a set of metal pins, each of which is dipped into wells containing different DNA samples. These pins are then used to deposit a small amount of these DNAs onto the surface of coated microscope slides in serial order with a distance of 100 μm to 200 μm between each DNA spot, thereby generating a high-density (up to 80,000 spots) microarray slide. Alternatively, inkjet technology has been successfully used to print oligonucleotides onto glass slides, which are then used for microarray hybridization. Another widely used array format, the Affymetrix GeneChip® (Affymetrix, Santa, Clara, CA), is generated on a quartz wafer by photolithography, which is the same process that is used in semiconductor manufacturing (Figure 2.2). One can think of the Affymetrix GeneChip® construction as in situ oligonucleotide synthesis on the chip. Initially, a mask is aligned with the wafer, and light is directed through the mask to activate (deprotect) exposed substrate. Next, chemically modified nucleosides are introduced and chemically coupled, and then a capping step blocks uncoupled sites. This process is repeated with different masks until the probes are synthesized to full length (20 to 25 nucleotides in length) to generate high-density microarrays.

To generate probes for spotted arrays, RNA samples isolated from two different cell types, embryonic stages, tissues, or treatments are reverse transcribed and labeled with either of two fluorescent molecules, Cy3 (green) or Cy5 (red) (Eisen and Brown, 1999). The labeled cDNAs (probes) are denatured, mixed, applied to the microarray containing glass slides, and allowed to hybridize competitively. Hybridized slides are washed and subjected to laser

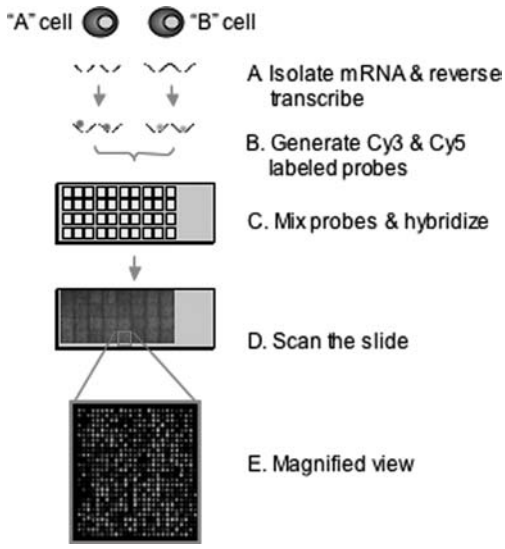
"A" cell
"B" cell
A. Isolate mRNA & reverse transcribe
B. Generate Cy3 & Cy5 labeled probes
C. Mix probes & hybridize
D. Scan the slide
E. Magnified view

FIGURE 2.1 **The basic principle behind spotted microarrays. A,** RNA is extracted from a cell or tissue sample and then converted to cDNA. **B,** Fluorescent tags (usually Cy3 and Cy5) are enzymatically incorporated into the newly synthesized cDNA, or they can be chemically attached to the new strands of DNA. **C,** The dye-labeled cDNAs are mixed and hybridized to the microarray, on which DNA has been spotted. **D,** A dye-labeled cDNA species that contains a sequence that is complementary to one of the single-stranded probe sequences on the array will hybridize to the corresponding spot. After hybridization, the red and green fluorescent signals from each spot are measured using a confocal laser scanner. The intensities indicate the level of expression of a particular gene. The ratio of red to green reflects the relative expression of each gene between the experimental and reference samples. **E,** A magnified view of panel C. (See color insert.)

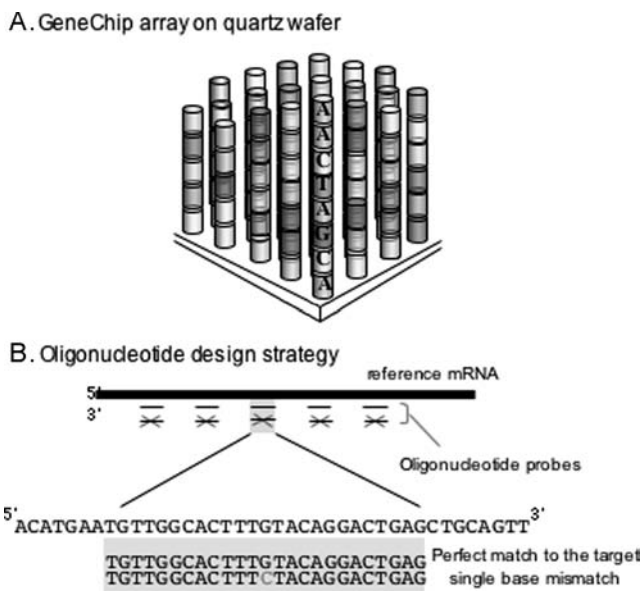

FIGURE 2.2 **The basic principle behind Affymetrix GeneChips®. A,** Millions of distinct oligonucleotides are directly synthesized on the surface of a quartz wafer by photolithography. About 20 to 25 distinct oligonucleotides, which are printed as individual features, represent the partial sequence of one gene. **B,** Perfectly matched and single base mismatched oligonucleotide probes against reference mRNA are designed. Each gene on the chip is typically represented by several different probe pairs on the chip. Scanned arrays produce raw data that consist of the intensities of the individual probe pairs (i.e., the perfectly matched and mismatched probes).

excitation, and then the resultant fluorescence of each dye is measured. The relative abundance of each mRNA present in the two samples being compared is inferred by measuring the ratio of the red and green fluorescence intensities for each spot on the array. The binding of specific fluorescent probes indicates whether genes are expressed. The intensity of the signals provides the abundance of the transcripts relative to control (frequently referred to as the "reference" probe; see later section) (Figure 2.1). Affymetrix GeneChips® use a single probe for each chip. A biotin-labeled cDNA probe is prepared for hybridization. After hybridization, the chip is stained with streptavidin–phycoerythrin to detect the probe; it is then washed and scanned with a confocal laser, and the distribution pattern of signal in the array is recorded.

B. Advantages and Disadvantages of Different Array Systems

Spotted microarrays are useful for comparing relative mRNA expression levels between two populations of cells. For instance, if the main goal is to identify genes that are up- or down-regulated after a particular treatment (e.g., growth-factor stimulation), one would label and simultaneously hybridize probes prepared from both a reference control (unstimulated) sample and from a growth-factor–treated sample. Microarray spots preferentially fluorescing for the treated sample probe represent growth-factor upregulated genes and, likewise, spots preferentially fluorescing for the untreated control probe represent genes that are downregulated by the growth factor.

Although this approach is useful for rapidly identifying changes in gene expression within slides, the experimental design does not allow for the easy comparison and analysis of expression levels among multiple different samples (experiments). For example, it is not straightforward to compare changes in gene expression (between control and treated samples) over multiple time points to chart temporal responses after a defined alteration to cells or their environment. To alleviate the problems of this type of analysis, one can design each hybridization experiment using a common reference sample. In the example above, the reference would be used with all time points. Thus, it is possible to indirectly compare the expression levels of two samples that are measured separately on two different slides. The ideal common reference should ensure consistent and nonzero values for all probes on the array so that no information is lost when the fluorescence ratios are calculated. For this purpose, mRNAs from whole embryos pooled from several developmental stages are frequently used. This ensures that mRNAs corresponding with each and all cDNA spots are represented at some level. This internal reference allows for the direct comparison of array experiments performed across tissues and different developmental states. Alternatively, hybridization can be spiked with a known amount of specific probes throughout the experiments, and the overall intensity can be normalized on the basis of the hybridization efficiency of the spiked probes. Methods are now available for standardizing global gene expression analysis among different platforms (Bammler et al., 2005).

Advantages of spotted cDNA microarrays include their relative affordability and increased detection sensitivity resulting from longer target sequences, which enhance the hybridization efficiency. Disadvantages include the difficulties associated with monitoring the expression levels of differentially spliced transcripts from a single genomic locus and distinguishing among closely related genes that may potentially cross hybridize. These handicaps do not exist

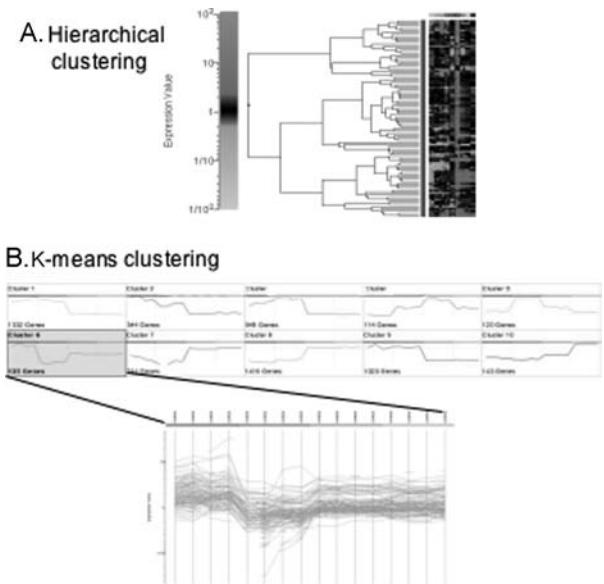

FIGURE 2.3 **Clustering analyses. A,** The hierarchical activity performs a clustering analysis on the basis of pairwise comparison between expression values. All pairs of genes are computed to identify the level of correlation between experiments. Genes or/and groups are connected with lines and presented as dendrograms, which are trees that are based on the similarity of their gene expression patterns throughout numerous experiments. Then, the next most similar groups are connected with a line and correlated with their nearest neighbors. Gene expression levels are shown in a color gradient. **B,** In K-means clustering, the number of clusters (K) is first assigned, and here a K-value of 10 was used. K-means clustering finds 10 clusters of genes with similar expression profiles. Genes giving a similar expression profile to cluster 6 are shown in the bottom panel. (See color insert.)

in oligonucleotide arrays, including Affymetrix GeneChips®. Oligonucleotide probes are designed to be relatively short and perfectly complementary to a target gene sequence. Therefore, a mismatched cDNA probe will not anneal efficiently to the oligonucleotide sequence. Additionally, in some cases, oligonucleotide chip design purposely incorporates a single base mismatch in the central region (Figure 2.3, B). Bona fide hybridization is distinguished from cross hybridization by comparing the signal obtained from the perfectly matched probe with that of the one mismatch probe. This sequence mismatch strategy, along with the use of multiple sequences for each gene, increases the specificity of the signal and minimizes the effects of nonspecific hybridization (Lipshutz et al., 1999).

Although microarray technology is a powerful addition to "genomics" research, gene expression analysis in eukaryotic cells poses a significant challenge to the current microarray technology as a result of complexity and sensitivity issues. For example, human cells are thought to express 80,000 to 100,000 different transcripts derived from only 20,000 to 25,000 estimated genes. Of these, 99% are rare, occurring at a frequency of less than 1 copy per 20,000 transcripts, and half of mRNA populations in a cell are the product of about 300 genes (Bishop et al., 1974; Davidson and Britten, 1979). To detect both the rare and abundant messages at the same time, microarray technology must be both sensitive and have a large enough dynamic range. Using current

technology, typical cDNA arrays can reliably detect mRNA levels equivalent to 1 transcript in 300,000 using conventional direct incorporation of fluorescent dyes.

The amount of RNA required to perform microarray analysis is often a limiting factor. Small sample sizes can make the recovery of sufficient mRNA difficult and thus prevent the synthesis of sufficient amounts of fluorescent cDNA probe. To overcome this limitation, synthesized cDNAs must first be amplified. Several different technologies are available for cDNA amplification. Examples include an adaptor-ligation–mediated PCR amplification method that allows one to obtain enough RNA from single cells to perform DNA microarray analysis. However, a major concern of such amplification is the fidelity of the representation of the original RNA population in the resulting amplified material.

C. Data Analysis and Bioinformatics

Microarrays have many variable experimental steps, including the design of the experiments themselves, RNA collection, probe preparation and labeling, and slide hybridization and scanning. Without the proper controls and quality checkpoints, the resulting data has the potential of being highly variable with an excess background level. When they are not properly monitored and controlled, these variations will drastically affect further data analysis and interpretation. In the past, the lack of standardization in arrays has presented problems during the exchanging and comparing of the array data sets. To facilitate this process, the scientific community has adopted the "Minimum Information About a Microarray Experiment" (MIAME) standard for describing a microarray experiment (Brazam et al., 2001). The MIAME standard has been adopted by many journals as a requirement for the submission of papers that incorporate microarray results.

When handling microarray data, several issues should be carefully considered (Church, 2002; Stoeckert et al., 2004). A large set of spot identification information and acquired array hybridization data must be organized and cataloged into a usable form. Because tens of thousands of data points are acquired simultaneously, the reliability of the array data (minimizing the randomness) needs to be confirmed so that only reproducible data can be processed during subsequent data-mining analyses. Otherwise, poor-quality data could poison the array analysis, and faulty interpretations could then be made. Before actual array data analysis can be conducted, raw data must go through a "normalization" process to determine the quality of the data sets. This process attempts to compensate for technical differences among chips. For example, because the incorporations of Cy3 and Cy5 dyes are often different among probe samples, to properly compare the signal-intensity data between reference and experimental conditions, a global normalization ("correction") process must be applied to the data sets to equalize the mean values of the expression levels for all genes between the experimental and control samples. Normalized data are compared using an analysis of variance test, which measures the difference between the means (averages) of two or more groups. Alternatively, a Bayesian probabilistic statistical method based on the *t* test can be used for comparing gene expression differences among different samples (Long et al., 2001).

Statistically treated data can then be clustered using a variety of different methods (Quackenbush, 2001). We will discuss two commonly used

approaches: hierarchical clustering and K-means clustering. Hierarchical clustering is the most popular method for microarray data analysis. In hierarchical clustering, genes with similar expression behaviors shown in numerous microarray experiments are grouped together and connected by a series of branches, which produces a dendrogram (or clustering tree; Figure 2.3, A). With this bottom-up method, experiments are grouped together to identify genes that may behave similarly or that have been coexpressed throughout experiments. K-means clustering is an algorithm to classify a given data set through a certain number (K) of clusters (Figure 2.3, *B*). Because the number of group sets must be determined before an analysis can be initiated, this can be considered to be a top-down approach. One of the powerful outcomes of these clustering analyses is the possibility to infer the probable functions of new genes based on similarities in expression patterns with those of known genes or to link unsuspected genes to specific biological responses (e.g., developmental function, occurrence of disease states). For example, coregulated genes may have tight relationships (i.e., similar expression patterns) because they are regulated by the same transcription factor or by the same signaling pathway. This ability to infer the activity of the gene by microarray screening is one of the strengths of microarray technology, and it is a major difference from traditional hypothesis-driven approaches.

II. APPLICATIONS OF DNA MICROARRAY TECHNOLOGY

A. Gene Expression Profiling by Microarrays

Microarray-based studies attempt to monitor transcript levels in differentiating cells, genetic mutants, complex diseases, and others. Microarrays have been successfully used to link a previously unsuspected gene to a particular disease. For example, by comparing and following the diagnosis of hundreds of tumor samples, marker genes linked to metastatic tumors were isolated (Laura et al., 2002). In another case, examining genes that were differentially expressed between insulin-resistant and normal strains of rat identified a gene linked to the regulation of glucose metabolism (Aitman et al., 1999). Finally, by examining gene-expression profiles in a family affected by sudden infant death syndrome, a gene linked to the disease was discovered (Puffenberger et al., 2005). A number of clinical trials using microarrays are currently underway for the prognosis and therapeutic guidance of these and other diseases.

Microarrays are also useful for identifying genes that respond to specific perturbations. For example, cells or embryos may be challenged with a growth factor or a chemical, and genes that are induced or inhibited can be identified. Gain-of-function studies involving mRNA overexpression and loss-of-function (knockdown) studies involving antisense morpholino oligonucleotides, siRNA, or the expression of dominant-negative variants of proteins will assist with the rapid identification of the genes that are affected by such manipulations. This will lead to the identification of potential gene function and the discovery of downstream target genes (Piano et al., 2002).

It is interesting to note that, although an enormous number of microarray studies have been performed so far, only a fraction have concerned developmental biology. This disparity was partly the result of the limited availability of high-quality, high-density microarray chips for some model organisms

together with difficulties in isolating sufficient amounts of RNA to carry out microarray experiments. An additional layer of complexity is added, because developmental biologists prefer to study gene expression in populations of identical cells rather than in a whole tissue, an organ, or another heterogeneous population of cells so that cell fate changes can be better studied. One ingenious way to overcome this difficulty is to use RNA isolated from green-fluorescent-protein–marked tissues, from cells of transgenic embryos, or from cells separated by fluorescence-activated cell sorting using antibodies to cell-specific surface antigens. Because cell sorting is unlikely to produce enough cells to perform a traditional microarray experiment, RNA extracted from purified cells must be amplified.

B. Comparative Genomic Hybridization Using Tiling Arrays

Microarrays are useful for studying DNA variants, with the primary application being the identification and genotyping of mutations and polymorphisms. These applications pose different challenges than monitoring RNA expression does, because the focus is not the quantitation of the transcripts but rather discriminating a single nucleotide mismatch on the basis of differential hybridization. For this reason, oligonucleotide arrays (as opposed to spotted arrays) have been the method of choice. A tiling array consists of short overlapping oligonucleotides that are "tiled" across a sequence of interest and that differ only by having a nucleotide substitution at a single position. An amplified cDNA product containing the expected sequence will hybridize best to the exactly matched probe, whereas the rest of the probes will hybridize weakly as a result of sequence mismatches. This type of relatively simple tiling array has been successfully used to map single nucleotide polymorphisms (SNPs; pronounced "snips"). This technology is seeing increased use in new applications, such as comparative genomic hybridization and chromatin immunoprecipitation (ChIP), which are both discussed in more detail later in this chapter.

C. Genotyping Single Nucleotide Polymorphisms

More than 99% of the genome sequence is identical across the human population. SNPs reflect the small sequence variations (often a single base change) that can occur within an individual gene. It is important to note that SNPs do not change much from generation to generation. Human genome sequencing projects have identified more than 2 million SNPs as genetic markers. Most SNPs are found outside of coding sequences, but some SNPs found within a coding sequence are of particular interest to researchers, because the change may alter the biological function of a protein. Because of the enormous potential to associate SNP maps with the development of complex diseases such as cancer, Alzheimer's, diabetes, and hypertension, researchers are feverishly working to identify thousands of useful SNP markers. For example, SNPs in the breast cancer genes 1 and 2 are associated with the development of breast cancers (Freedman et al., 2005). SNPs in the apolipoprotein E gene have been linked to a higher risk of developing Alzheimer's disease (Bullido et al., 1998). One of the goals in this field is to generate arrays that are capable of genotyping thousands of polymorphisms in a single hybridization. This may someday allow for the construction of an individual's genetic fingerprint that will be able to predict individual risk for developmental disorders and diseases. After

this technology emerges, we can also anticipate the discussion of social and ethical issues associated with the use of this information.

D. Comparative Genomic Hybridization

The completion of various genome projects coupled with improved array printing technology has led to the generation of more sophisticated tiling arrays. A new generation of high-density tiling array methods use millions of DNA probes that are evenly spaced across the genome, including in both coding and noncoding regions. Some of the genome tiling arrays consist of a multiple-array set, with each set containing more than a few million probe pairs. These oligonucleotide probes, which usually ranging in size from 25 to 50 nucleotides long, are tiled at a discrete resolution (e.g., 10 nucleotides apart) to cover the entire genomic sequence (Ishkanian et al., 2004). To perform a typical tiling array experiment, total genomic DNA is isolated from test and reference cell populations, fragmented by shearing, and then differentially labeled and simultaneously hybridized to DNA microarrays. The relative hybridization intensity of the test and reference signals at a given location should be proportional to the relative copy number of those sequences in the test and reference genomes. If the reference genome is normal, then increases and decreases in the intensity ratio directly indicate DNA copy-number variation in the genome of the test cells. This type of microarray experiment (Mantriparagada et al, 2004), which detects chromosomal imbalances and variation in DNA copy number, is known as *comparative genetic hybridization,* and it would be useful for mapping the regions of a genome containing deletion mutations, chromosome translocation, and rearrangements.

E. Transcriptome Analysis

The transcriptome can be considered as the complete collection of transcribed elements of the genome. Broadly speaking, transcriptome mapping attempts to define regions of transcription, transcription factor binding sites, sites of chromatin modification, sites of DNA methylation, and chromosomal origins of replication. The tiling array covering both coding and noncoding regions has proven to be extremely valuable for transcriptome analysis. For example, probe sets on the normal microarrays used for gene expression profiling are based on information about known transcribed genes. This means that we can only analyze the genes that have already been isolated and that some genes that may have important functions but that have not yet been cloned will escape unnoticed. The tiling array will overcome this shortfall and detect novel genes or microRNAs with sequence information that is not yet available in expressed sequence tag databases. Additionally, tiling arrays can detect alternatively spliced variants of genes. An interesting finding that has emerged from transcriptome mapping is that large regions of the genome beyond the coding segments are often transcribed (Kapranov et al., 2002; Stolc et al., 2004; Katayama et al., 2005). This has provided new insights into the basic understanding of how transcriptional regulation may occur in animals and the function of so-called "junk" DNA. Scientists are currently pushing the limits of transcriptome research by building whole-genome tiling arrays that interrogate the genome at resolutions at the levels of individual nucleotides.

F. Chromatin Immunoprecipitation Analysis

ChIP detects interactions between specific transcription factors and the regions of genomic DNA to which they bind (Horak and Snyder, 2004; Ren and Dynlacht, 2004). The early generation of microarrays for ChIP analysis was produced by PCR-amplifying promoter regions of limited sets of genes. This powerful technology has been rapidly evolving after the completion of various genome projects. Current promoter arrays can range from spotting synthetic oligonucleotide arrays covering the upstream and downstream of tens of thousands of promoter regions to tiling arrays covering the entire genome at nucleotide resolutions.

For ChIP analysis, proteins are crosslinked to DNA by treating whole cells (or nuclei) with formaldehyde, and this is followed by the fragmentation of the protein-bound DNA by sonication or endonuclease digestion (Figure 2.4). Antibodies against a specific chromatin-associated protein are then used to immunoprecipitate protein–DNA complexes. This results in enrichment for fragments bound to the immunoprecipitated proteins. These fragments are amplified, and fluorescent-labeled probes are generated both from an experimental and a control sample; they are then hybridized to the promoter-array chips. For control, a parallel experiment can be performed after omitting the

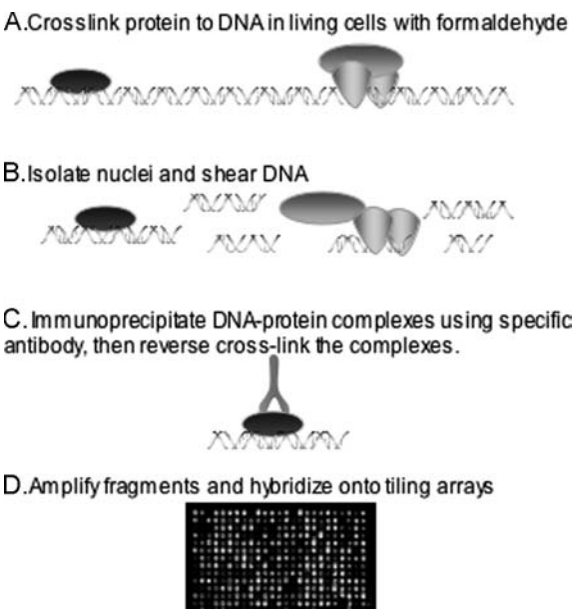
A.Crosslink protein to DNA in living cells with formaldehyde
B.Isolate nuclei and shear DNA
C. Immunoprecipitate DNA-protein complexes using specific antibody, then reverse cross-link the complexes.
D.Amplify fragments and hybridize onto tiling arrays

FIGURE 2.4 **ChIP-on-chip analysis. A,** The ChIP-on-chip method aims at determining the DNA sequences to which given proteins are bound. These binding sites may indicate the functions of various transcriptional regulators. **B** and **C,** Cells are treated with formaldehyde to immobilize protein–DNA complexes, which are later sheared by sonication and precipitated using specific antibodies against the protein of interest. **D,** After the removal of crosslinked samples, the DNA sample is subjected to PCR amplification. ChIP-enriched DNA and control genomic DNA samples are independently labeled with fluorescent dyes (Cy5 and Cy3). For a single-color scan, two different DNA pools are hybridized to two separate whole-genome tiling arrays, and the data are compared. For a two-color analysis, two differentially labeled samples are combined and hybridized to a single array. The DNA sequences enriched are thereby identified by hybridization on DNA chips. (See color insert.)

antibody or using nonspecific antibodies. By comparing signals between experimental and control (reference) probes, the enrichment of specifically bound fragments can be directly detected. The bound fragments are expected to reside adjacent to or within genes that are regulated by the specific transcription factor. Genome-wide ChIP-on-chip analysis is gaining popularity, and it has been successfully used to identify target genes regulated by individual transcription factors or in combination. For example, using ChIP-on-chip analyses of MyoD, Myogenin, and MEF2, it was possible to successfully construct a blueprint of a gene regulatory network for myogenic differentiation (Blais et al., 2005). Likewise, target gene networks have been identified for HNF transcription factors for hepatocyte and pancreas differentiation and for Oct4, Nanog, and Polycomb for stem cell differentiation (Odom et al., 2004; Lee et al., 2006; Loh et al., 2006). ChIP experiments were also employed to identify different histone modifications associated with active as compared with inactive chromatin (Bernstein et al., 2004). The key reagent for the success of this approach is the access to high-quality antibodies specific for a protein of interest.

III. CONCLUDING REMARKS

Microarray technology is valuable in the gene expression profiling of a variety of cell types. Although the technology has been predominantly used to study gene expression patterns, refined approaches enable scientists to address increasingly complex questions and to perform more intricate experiments. We envision that the data obtained from microarray work will become essential for the detection of various diseases and developmental abnormalities and for inferring the probable functions of new genes. Furthermore, applications of microarray to developmental biology will enlarge our understanding of the gene regulatory programs that govern embryogenesis, oncogenesis, and diseases that affect humans.

SUMMARY

- Microarrays are experimental tools that were originally designed to measure the levels of transcripts in different cells in response to experimental manipulation.
- Microarrays allow us to simultaneously examine the expression of thousands of genes, thereby permitting the linking of previously unsuspected genes to particular developmental processes and diseases.
- Microarrays are useful for the study of DNA variants, with the primary applications being the identification and genotyping of mutations and polymorphisms.
- The availability of whole genome tiling arrays allows us to detect SNPs, mutations, and chromosome abnormalities and to perform transcriptome mapping and ChIP-on-chip analyses.

ACKNOWLEDGMENTS

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GLOSSARY OF TERMS

ChIP-on-chip

Chromatin immunoprecipitation (ChIP) is an experimental approach for the identification of the transcription factors associated with specific regions of the genome. Chromatin associated with a specific protein or proteins is precipitated using appropriate antibodies. The enrichment of the DNA fragments relative to the input is measured at each genomic location on the basis of array hybridization.

Comparative genomic hybridization

Comparative genomic hybridization measures DNA copy number differences between two different samples using a spotted microarray or a high-density tiling array.

Microarrays

The technique explores the ability of DNA or RNA molecules to hybridize specifically to DNA-probe templates spotted on a glass slide or a quartz wafer. In a single experiment, the expression levels of hundreds or thousands of genes within a cell can be measured to determine the amount of mRNA bound to each site on the array.

Single nucleotide polymorphism (SNP)

A SNP is a small genetic change that occurs within a person's DNA sequence. SNPs may fall within coding sequences of genes, noncoding regions of genes, or in the intergenic regions between genes.

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3

CHEMICAL AND FUNCTIONAL GENOMIC APPROACHES TO STUDY STEM CELL BIOLOGY AND REGENERATION

WEN XIONG and SHENG DING

The Scripps Research Institute Department of Chemistry and the Skaggs Institute for Chemical Biology, La Jolla, CA

INTRODUCTION

Regeneration—a complex process involving the restoration of cells, tissues, and structures that are lost or damaged during disease, injury, or aging—is more commonly observed among the lower organisms (e.g., amphibians) than it is among mammals. Nevertheless, the regenerative process typically involves stem or progenitor cells. Stem cells are cells that can self-renew to produce themselves and differentiate to generate lineage-restricted progenies. They have the remarkable potential to develop into essentially all of the cell types found in the organism. Therefore, in addition to their potential therapeutic value, they provide excellent model systems for basic studies of cell behaviors. Tremendous efforts have been concentrated on the identification, isolation, and characterization of embryonic and tissue-specific stem cells in various organisms. More recently, the concept of cancer stem cells has been implicated and strengthened in a number of tumor types, and knowledge of stem cell biology may also provide new therapeutic strategies to cure cancers. Despite the enormous progress made so far, stem cell isolation, the maintenance of self-renewal, and directed differentiation remain challenging. Consequently, the realization of their therapeutic potential would require an improved ability to control their fate and a better understanding of the precise molecular mechanisms underlying their proliferation, differentiation, migration, and survival at the systems level. The completion of genome sequencing projects in several organisms in conjunction with advances in high-throughput

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technologies has allowed studies at the systems level. Genome-wide approaches have been emerging for various biological investigations, and they should ultimately provide us with precise information about the abundance and modification state of all molecules in a cell at a given time or under a certain condition. A small molecule approach has historically proved to be a useful tool for modulating cell fate and probing the underlying molecular mechanisms. The application of chemical and genomic approaches in stem cells will greatly advance our understanding of fundamental questions in stem cell and developmental biology, and it may ultimately facilitate the development of novel therapeutic strategies to treat human diseases or to stimulate tissue or organ regeneration *in vivo*. This chapter will focus on the current advances in the chemical and functional genomic approaches in stem cell research.

I. OVERVIEW OF STEM CELLS

A. Definitions of Various Types of Stem Cells

Stem cells are cells that have the ability to self-renew for long periods of time and to differentiate into specialized cell types in response to appropriate signals. Traditionally, stem cells are classified as either embryonic or tissue specific. Embryonic stem cells (ESCs) are typically derived from the inner cell mass of the blastocyst. They possess an unlimited capacity for self-renewal, and they have the potential (i.e., pluripotency) to develop into any cell types found in the three primary germ layers of the embryo (i.e., endoderm, mesoderm, and ectoderm), as well as germ cells and extraembryonic cells (Hubner et al., 2003; Toyooka et al., 2003; Geijsen et al., 2004). By contrast, tissue-specific stem cells are multipotent, and they are found in differentiated tissues. They are capable of self-renewal, but they generally can only differentiate into restricted cell types of the tissue from which they originate. These cells are believed to function as the "reservoir" for cell and tissue renewal during homeostasis or tissue regeneration. The most studied tissue-specific stem cells include hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), neural stem cells (NSCs), epidermal stem cells, and skeletal muscle stem cells. HSCs are probably the best-characterized somatic stem cells. They can be prospectively isolated from bone marrow, but they are difficult to expand without differentiation *in vitro*. HSCs have the capacity to provide the lifelong reconstitution of all blood–cell lineages after transplantation. Although the *in vivo* origin of MSCs remains elusive, MSCs are multipotent progenitor cells that can be isolated from multiple tissues, expanded substantially *in vitro*, and differentiated into a variety of cell types, including osteocytes, adipocytes, chondrocytes, skeletal muscle cells, and neurons (Pittenger et al., 1999; Dezawa et al., 2004; 2005). The discoveries of NSCs in the adult central nervous system (CNS) and their regenerative roles in brain damage have suggested approaches involving cell replacement therapy and the stimulation of *in vivo* regeneration for the treatment of neurodegenerative diseases and CNS injury. In contrast with HSCs, NSCs can be expanded in the presence of growth factors (e.g., basic fibroblast growth factor [bFGF]), but the noninvasive isolation and purification of significant numbers of NSCs from the brain remain challenging.

More recent evidence supports a longstanding notion that cancers are initiated and maintained by cancer stem or progenitor cells (Beachy et al., 2004). There is a growing body of evidence that suggests a close relationship between normal stem cells and cancer stem cells: the self-renewal mechanisms of normal stem cells and cancer stem cells are similar; the deregulation of the developmental signaling pathways involved in stem cell self-renewal is associated with oncogenesis; and tumors contain "cancer stem cells" that may arise from normal stem cells or progenitor cells via cellular transformations (Pardal et al., 2003). A better understanding of stem cell regulation will not only provide novel therapeutic approaches to tissue regeneration, but it may also help identify the molecular triggers and potential cures for various types of cancers.

B. Stem Cell Behaviors and Functions

As a result of their unique properties of self-renewal and differentiation into various mature cell types, stem cells not only provide great opportunities for studying tissue and organ development, but they also hold important therapeutic potentials for regenerative medicine. Encouraging therapeutic examples include the transplantation of HSCs or specific neural stem and progenitor cells for the treatment of hematologic diseases or Parkinson disease in humans, respectively. More efficient and highly selective methods of controlling stem cell fate for producing homogenous populations of particular cell types will be essential for the therapeutic use of stem cells; this will facilitate studies of the molecular mechanisms of development.

Stem cell fate is determined by both intrinsic regulators and the extracellular microenvironment (niche). It is postulated in one model that, to maintain the homeostasis *in vivo*, the stem cell undergoes asymmetric division, and the daughter cell that is proximal (i.e., still attached) to the "niche" remains undifferentiated whereas the one distal to the niche differentiates into a specific cell type (Kiger et al., 2001; Tulina and Matunis, 2001). After stem cells are isolated from where they reside and cultured *in vitro* outside of the "niche," they are likely to differentiate spontaneously into a heterogeneous population of cell types unless instructed by specific signals. Therefore, it is a great challenge to maintain stem cell self-renewal in culture and to induce homogenous lineage-specific differentiation. Stem cell expansion and differentiation *ex vivo* are generally controlled by culturing the cells in a specific configuration, either attached monolayer or suspended aggregates, with "cocktails" of growth factors, signaling molecules, or genetic manipulations. However, most of these conditions are either incompletely defined or nonspecific with regard to regulating the desired cellular process. Undefined conditions often result in an inconsistency in cell culture and heterogeneous populations of cells that would not be useful for cell-based therapy and that would complicate the biological study of a particular cellular process.

Consequently, current challenges of stem cell research remain in two areas: first, the lack of precise and highly selective methods for homogeneous stem cell self-renewal and differentiation into specific cell types, and, second, the lack of a complete understanding of these processes at the molecular level. Advances in both aspects will enable better control of stem cell fate and thus facilitate clinical applications. Chemical and genomic approaches—including genomics, functional genomics, and proteomics—have been undertaken to address the above challenges.

II. GENOMIC APPROACHES IN STEM CELL RESEARCH

A. Genome-Wide Expression Analysis

Any given state of a cell can be perceived as the phenotypic display of a unique gene expression pattern of that cell. Global expression analysis at the mRNA level, which is also referred to as *transcriptome profiling,* is a widely used method to assign molecular signatures to a given cell population or tissue. In addition, profile comparison between two distinct cell populations can identify the key molecules that contribute to the cell type differences or to a specific cellular process (e.g., self-renewal, differentiation). The main purposes of stem cell transcriptome profiling are twofold: to identify the signature genes that define the cell type (e.g., stemness) and to identify the genes that can maintain stem cell self-renewal or direct its differentiation to a specific cell type of interest.

Three major methods of transcriptome analysis—microarrays, short sequence tags, and expressed sequence tags—have been successfully applied in stem cells to discover signature and essential genes through the systematic comparison of profiles among different cell types and species (Fortunel et al., 2003; Sato et al., 2003; Sperger et al., 2003; Brandenberger et al., 2004; Ng et al., 2005). More recently, chromatin immunoprecipitation on a chip platform has been developed. This technology goes beyond gene expression to explore gene regulation and to determine the precise binding sites of proteins on DNA sequences (Boyer et al., 2005). As compared with the traditional one-gene-at-a-time approach, the transcriptome profiling approach greatly speeds up gene discovery, and it provides new ways of thinking about biological questions. However, there are several limitations associated with genome-wide expression analysis:

1. False positives and negatives could be mixed in every transcriptome data set as a result of experimental, technical, or analytical flaws;
2. It is difficult to draw a causal relationship between a gene's expression and a phenotype;
3. Follow-up studies of transcriptome profiling, including loss- and gain-of-function studies, typically still require the conventional one-gene-at-a-time approach;
4. Gene regulation achieved at the posttranscriptional, translational, and posttranslational levels also play significant roles in a cellular event, and these are not reflected in the transcriptome profiling; and
5. The dynamics of protein function affected by cellular localization, degradation rate, protein–protein interactions, and so on can only be determined at the protein level.

Additional approaches, including functional genomics and proteomics (as described later) are needed to complement the transcriptome genomics approach.

B. Functional Genomics

Functional genomics aims not only to determine the complex roles of vertebrate genes during development but also to screen for molecules that are involved in a biological process on a genome-wide scale. There are two main approaches in the functional genomics that are currently applied to stem cell research: gene trap and high-throughput screens. Gene trap was designed for

the systematic knockout of gene function on a genomic scale to determine the roles of mammalian genes during embryonic and postembryonic development, but recently it has also been modified for functional screens during stem cell development. A more important advancement, however, has been the application of high-throughput screens, in which functions of a genome-scale collection of genes are simultaneously evaluated in a biological process.

1. Gene Trap

Gene trap is a form of random intragenic insertional mutagenesis that was designed to perturb gene function. There are promoter and enhancer trap and polyadenylation trap systems. An important feature of gene trap is that the disrupted gene can be easily identified by the rapid amplification of cDNA ends. As compared with the targeted gene inactivation approach based on homologous recombination, gene trap is simple, rapid, and cost-effective. However, as a result of the diploid nature of the mouse genome, recessive mutations created by gene trap cannot elicit phenotypic consequences to reflect the function of trapped genes unless homozygous transgenic mice are generated. A sophisticated system has been developed recently to circumvent this problem (Guo et al., 2004). Taking advantage of the highly efficient mitotic recombination in blm-deficient ESCs, a genome-wide library of homozygous mutant cells was generated by gene trap, which enabled a direct phenotypic genetic screen in ESCs. Often, a reporter gene is also included in the trapping vector to capture the endogenous expression pattern of the disrupted gene. ESC clones carrying traceable insertional mutations can be assayed *in vitro* for reporter gene activity under various cell lineage specification conditions, which allows for the identification of developmentally regulated genes. A collection of mouse mutations has been generated by gene trap and organized in a searchable database (To et al., 2004). To facilitate the functional categorization of trapped genes, a responder mouse ESC (mESC) line carrying a dominant selection marker has been used (Chen et al., 2004). Recently, gene trap has also been carried out in human ESCs (hESCs; Dhara and Benvenisty, 2004) and adult rodent neural progenitor cells (Scheel et al., 2005).

2. High-Throughput Screening Technologies

Conventional genome-scale functional screens have been performed largely on libraries of pooled cDNA clones. As a result of the complexity of such libraries and the need to oversample them to find the rare clones, the assay is often limited by exceedingly simple readouts (which is typically a selection method), and it requires the subsequent deconvolution of clone identities. A pluripotency-associated master gene, Nanog, has been discovered using this technique (Mitsui et al., 2003). With recent advances in automation and detection technologies for high-throughput screening and the development of individually arrayed molecular libraries (cDNAs, siRNAs, miRNA, or small molecules, each of which has members that are spatially separated in different wells of multiwell plates), more complex assays can be used for real functional screens instead of selection, such as monitoring morphological changes or dynamic cellular events without the need for clone rescue and deconvolution. In contrast with the typical random mutagenesis screen, the high-throughput functional screen has several advantages: first, the identities of the hits are already known; second, the genome can be saturated; and

third, the maintenance and replication of the screen are much more convenient and efficient. There are generally two types of high-throughput functional screens based on the assay used: reporter activity-based screens and high content phenotype-based screens. Hits identified are further characterized using traditional biochemical and cellular methods.

3. Large-Scale Genetic Approaches: cDNA and RNAi Libraries

Perturbation of gene function can be achieved by overexpression (cDNA) or gene knockdown. RNA interference (RNAi), which is a highly conserved gene-silencing event that functions through the targeted destruction of the individual mRNA with the introduction of a homologous double-stranded RNA (dsRNA), is a powerful tool to knock down gene expression. Both vector-encoded short hairpin RNAs (shRNAs) and chemically synthesized double-stranded siRNAs have been demonstrated to be effective RNAi tools (Elbashir et al., 2001; Paddison et al., 2004). Screens using both cDNA and shRNA libraries have been successfully carried out in mammalian cells (Elbashir et al., 2001; Michiels et al., 2002; Paddison et al., 2004;). Recently, proof-of-concept screens using an arrayed synthetic siRNA library targeting more than 5000 human genes have been carried out in hMSCs to identify the endogenous repressors of osteogenic or adipogenic specification, which, upon silencing, could initiate the differentiation of hMSCs into osteoblasts or adipocytes, respectively. Such screens yielded a large number of novel hits and provided a foundation for studying the genetic network that controls self-renewal, the osteogenesis and adipogenesis of hMSCs, and, potentially, the molecular rationale for treating certain bone and metabolic diseases.

However, the cost and effort required to generate the arrayed libraries and the availability of screening technology have been constraints. The development of cell microarrays, which use a microarray format to substitute for the multiwell plate format, could potentially drive down the cost of high-throughput studies; however, this technique is still in its infancy (Wheeler et al., 2005). In addition, there are significant technical challenges to applying the arrayed high-throughput screening technologies in primary cells and stem cells, which are more difficult to transfect and susceptible to side effects when transfection methods are used. Gene delivery methods based on viral transduction may provide an alternative solution to this problem (Michiels et al., 2002; Berns et al., 2004). Furthermore, on the basis of the identified putative "stemness" genes or differentially regulated genes during stem cell differentiation through transcriptome profiling, selected cDNA or siRNA clones could be generated and collected to systematically evaluate the function of these hits in relevant biologic contexts. Although high-throughput screening is a highly productive and promising technique to complement transcriptome profiling, perturbation of a single molecule may often not be sufficient to induce a particular biologic event. In this case, sensitized screen conditions will need to be sought out. Moreover, the careful design and validation of constructed libraries and screen assays will be essential, especially in the case of RNAi, because off-target effects would be hard to pursue.

C. Proteomic Technologies and Mass Spectrometry

There has been tremendous interest in developing and applying protein profiling technologies for examining protein–protein interactions and protein

activities. More conventional tools (e.g., yeast two-hybrid and protein microarray systems) are limited as a result of sample analysis in artificial environments and their inability to identify interactions that are stabilized by more than two partners. Mass spectrometry (MS) enabled by new instrumentation coupled with various protein-separation techniques has emerged as a driving force in proteomics for analyzing protein abundance and modifications. Although protein tagging and pull-down followed by MS present a generic approach for the analysis of protein complexes, the two-dimensional difference gel electrophoresis (2D-DIGE) separation of proteins labeled with fluorescent dyes and the capillary separation of proteins labeled with isotopes (SILAC and ICAT) allow for comparative protein quantification by MS.

1. Fluorescent-Dye–Labeled 2D-DIGE

Two-dimensional electrophoresis (2-DE) separates complex mixtures of proteins (e.g., whole-cell lysates) according to isoelectric point and molecular mass. 2-DE data reflect changes in protein expression level, isoforms, and posttranslational modifications. Advances in prefractionation methods and the application of narrow-overlapping immobilized pH gradients yield a greater resolution of protein spots and sensitize the detection of low-abundance proteins, as demonstrated in the attempt at developing a comprehensive proteomic analysis of undifferentiated murine R1 ESCs using 2-DE contigs (Elliott et al., 2004). Of the proteins resolved from the 2-DE contigs, a large proportion was identified as DNA repair enzymes in addition to ribosomal, transcriptional, and translational proteins. These findings may reflect the properties of ESCs to resist DNA damage while maintaining the undifferentiated state and to quickly change phenotype as seen during differentiation. Quantification data from 2-D gels initially relied on the intergel comparison of sample populations using traditional stains (e.g., silver, Coomassie blue) with low dynamic ranges. Intragel comparisons can now be accomplished with the assistance of Cy dyes, which confer greater detection sensitivity and allow for the multiplexing of samples. Because fluorescent Cy dyes have the same chemical reactivity but distinct excitation and emission spectra, multiple samples can be run on the same gel, and relative protein abundance can be assessed from differential fluorescence intensities. Using this technique, a comparative proteomic analysis between two hESC populations with differential motility was performed (Evans et al., 2004). The greatest strength of 2-DE is its ability to distinguish proteins with varying posttranslational modifications (e.g., phosphorylation, glycosylation, ubiquination). Advancements in protein separation and labeling in 2-DE technology combined with MS have enabled the generation of large-scale proteome profiles to occur and provided a more reliable means of collecting relative protein expression data. Detection sensitization will require further improvements in staining sensitivity and sample preparation to unmask the "unseen proteome."

2. Capillary Separation with Isotope Labeling

2D-DIGE has been the traditional means for quantitative proteomic data collection, but the limitations of protein compatibility with gel electrophoresis have motivated efforts to develop capillary-based separation techniques, such as capillary liquid chromatography and capillary electrophoresis. The benefits

of capillary-based separation over 2-DE are its dynamic range and sensitivity. 2-DE requires large amounts of the sample to visually detect protein spots, whereas capillary-based separation requires a very small amount of sample with greater detection sensitivity. Recently, a large-scale identification of protein expression in mESCs was carried out (Nagano et al., 2005). Using automated 2D liquid chromatography coupled with MS analysis, proteins—including transcription factors characteristic of ESC and those previously reported as ESC-specific or "stemness" genes—were identified.

New techniques that have granted quantitative information to be derived from capillary-based separation are isotope-coded affinity tag (ICAT) and stable isotope labeling by amino acids in cell culture (SILAC; Gygi et al., 1999; Ong et al., 2002). The difference between ICAT and SILAC is the means by which isotopes are added to the protein mixtures. In SILAC, mammalian cells are cultured in media that lack a standard essential amino acid but that are supplemented with a nonradioactive isotopically labeled form of that amino acid. Over time, the labeled amino acid is completely incorporated into the proteome. In ICAT, isotopes are chemically added to samples by covalent attachment to each cysteinyl residue. After chemical incorporation, ICAT samples require an additional affinity step to collect only labeled proteins for MS analysis. SILAC is a simple, cost-effective approach to quantitative proteomics, but it requires cells to be alive and cultured until the isotope is completely incorporated. Alternatively, ICAT can be applied to both living and dead cells, but it depends on the presence of cysteinyl residues. After the isotopes have been completely incorporated into samples either via ICAT or SILAC, samples labeled with various isotopes are first mixed together and then analyzed together with MS. MS data give not only multiple peaks per peptide (corresponding with the relative heavy and light isotope samples), but they also give comparative protein abundance that is based on peak amplitudes. This technology can be used to observe differences between different developmental stages (Kratchmarova et al., 2005). SILAC with MS was employed to comprehensively compare proteins that were tyrosine phosphorylated in response to epidermal growth factor and platelet-derived growth factor (PDGF) for the purpose of deriving the basis of the differential induction of hMSCs into bone-forming cells by epidermal growth factor but not by PDGF. Although the types of signaling proteins that are modified during stimulation by both ligands largely overlapped, the phosphatidylinositol 3-kinase pathway was exclusively activated by PDGF. Chemical inhibition of this pathway allowed for the PDGF-induced osteogenic differentiation of hMSCs. This work illustrates the ability of quantitative proteomics to discover critical differences that are capable of changing cell fate by directly comparing two differential MS protein profiles.

III. CHEMICAL TECHNOLOGIES IN STEM CELL STUDIES

Cell-permeable small molecules that can modulate the function of specific proteins with exquisite precision provide convenient and efficient spatial and temporal control of gene function in a biological system, and they are powerful tools that complement genetic techniques. Small molecules, such as dexamethasone (a glucocorticoid receptor agonist), ascorbic acid, 5-azacytidine (5-aza-C; a DNA demethylation agent), trichostatin A (an histone deacetylase

inhibitor), and *all-trans* retinoic acid (RA), have proven to be extremely useful for modulating and studying the differentiation of various stem cells. For example, studies with 5-aza-C, which induces the myogenic differentiation of a mouse mesenchymal progenitor cell line, led to the discovery of a master transcription factor, MyoD, that is responsible for skeletal myogenic fate determination. Although such chemical tools have historically been used to investigate biological systems, advancements in chemical synthesis, high-throughput screening, and molecular profiling technologies have rejuvenated chemical approaches in biology. Chemical libraries composed of millions of discrete compounds can be efficiently generated, assembled, and "mined" through high-throughput functional screens. Screening for compounds that generate a desired phenotype in cells or animals and then characterize their mechanism of action may thus serve as an alternative approach for identifying key players in a biological process.

A. Rational Design of Chemical Libraries and Combinatorial Technology

1. Chemical Libraries

One approach to generating functional small molecules that control stem cell fate involves the use of phenotypic or pathway-specific screens of synthetic chemical or natural product libraries. The size and diversity of a given purified chemical library as well as the selection method determine the chance of finding a desired "hit" compound. With recent advances in automation and detection technologies, millions of discrete compounds can be screened rapidly and cost-effectively. However, although combinatorial technologies allow for the synthesis of a large number of molecules with immense structural diversity, it is impossible to saturate the chemical space. Because the diversity of chemical libraries is largely constrained by the synthetic tractability, new synthetic technologies are the driving force to expand current chemical diversities for filling the chemical space. In addition, introducing a high level of structural variability to increase the molecular diversity of a chemical library drastically reduces the average fitness of the library to a given biologica selection or screen, thereby resulting in most molecules being inactive (analogous to population genetics). Consequently, the careful design of a chemical library becomes a critical aspect of combinatorial synthesis (diversity vs. fitness).

The concept of "privileged structures" describes selected structural motifs that can provide potent and selective ligands for multiple biologic targets by introducing different substitutions onto the same scaffold. Privileged structures typically exhibit good "drug-like" properties, such as good solubility, membrane permeability, oral bioavailability, and metabolic stability, which make the further development of "hits" into "leads" less problematic. Given the success of privileged structures, the diversification of these scaffolds using combinatorial techniques provides not only large numbers of compounds but also highly enriched "functional" molecules. Using key biologic recognition motifs as the core scaffolds may represent one of the most straightforward and productive ways to generate "privileged" chemical libraries. Previously, we developed the concept of using privileged molecular scaffolds themselves as a diversity element for combinatorial synthesis (Ding et al., 2001; 2002a; 2002b; Wu et al., 2001) to maximize the diversity while retaining a minimal threshold of fitness to biological screens. With this approach, a variety of

naturally occurring and synthetic heterocycles that are known to interact with proteins involved in cell signaling (e.g., kinases, cell surface and nuclear receptors, enzymes) were used as the core molecular scaffolds. These included substituted purines, pyrimidines, indoles, quinazolines, pyrazines, pyrrolopyrimidines, pyrazolopyrimidines, phthalazines, pyridazines, pyridines, triazines, and quinoxalines (the first diversity elements). A general synthetic scheme (Figure 3.1) was then developed that could be used in parallel reactions to introduce a variety of substituents into each of these scaffolds to create a diverse chemical library. The library synthesis involved introducing a second diversity element into these heterocyclic scaffolds using solution-phase alkylation or acylation reactions. This was followed by the capture of the modified heterocycles onto solid support using different immobilized amines (introduced as a third diversity element). The resin-bound heterocycles could then be further modified (introduced as a fourth diversity element) through a variety of chemistries, including acylation, amination, and palladium-mediated cross-coupling reactions with amines, anilines, phenols, and boronic acids. Using these chemistries in conjunction with the "directed-sorting" method, we have generated diverse heterocycle libraries consisting of more than 100,000 discrete small molecules (representing more than 30 distinct structural classes), with an average purity of greater than 90%. These libraries have been proven to be a rich source of biologically active small molecules targeting various proteins involved in a variety of signaling pathways.

2. High-Throughput Screens

To systematically identify small molecules that can generate a cellular phenotype of interest, high-throughput screens of these large and diverse chemical libraries are carried out in a desired model system, such as a cell line (Figure 3.2) or a simple organism (e.g., Xenopus, zebrafish) with an appropriate readout, such as luminescence (e.g., a luciferase reporter), fluorescence (e.g., an enhanced green fluorescence protein [eGFP] reporter), or absorbance (e.g., enzymatic reactions to generate chromophores). However, such assays only provide limited information, and they require a battery of secondary assays to determine the precise cellular pathways or processes being affected. With recent advances in high-content imaging technologies (e.g., autofocus and image analysis), high-resolution microscopy/image-based screens allow for the capture of multiple parameters from a single reading at the single-cell level, thereby facilitating the identification of molecules with a desired biologic activity.

3. Lead Optimizations

Typically, the initial hits from the primary cellular screens may not be ideal for probing their mechanism of action (MOA) using affinity-based and functional genomic approaches or for serving as clean research tools in biological assays both *in vitro* and *in vivo*. Consequently, there is a need to improve the hit compound's properties (e.g., potency, specificity, solubility, bioavailability) via detailed structure-and-activity-relationship studies. These involve reiterated rounds of testing structurally related compounds that are modified via medicinal chemistry around substituent and scaffold. Such studies may also identify a linkage position on the molecule for attachment to a solid support without adversely affecting its activity (for affinity pull-down assay).

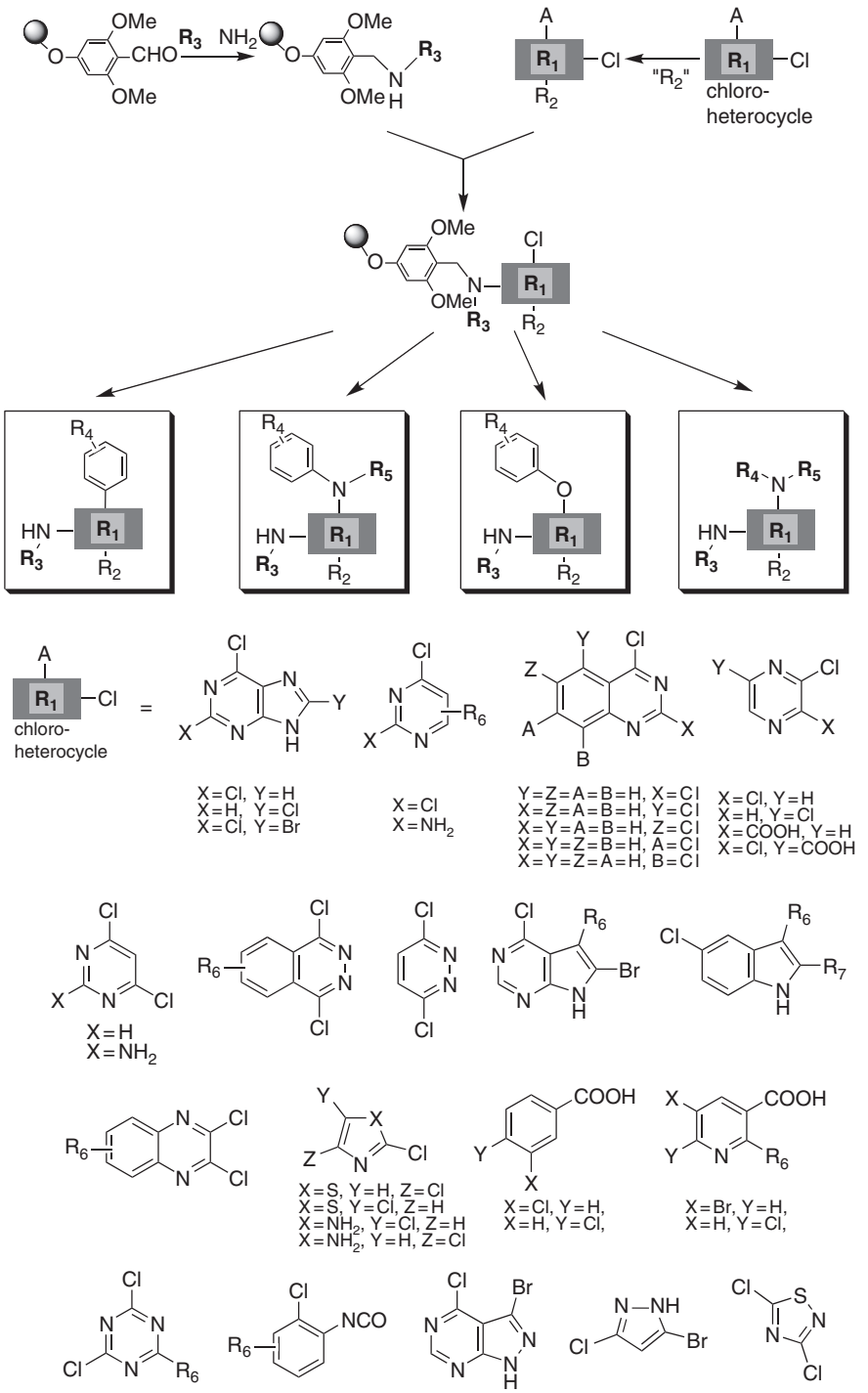

FIGURE 3.1 Combinatorial synthesis of heterocyclic library.

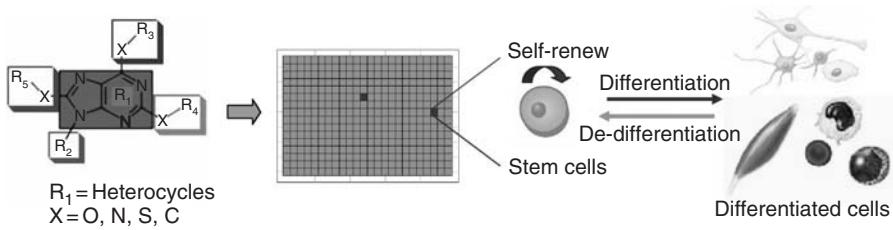

FIGURE 3.2 **Cell-based high throughput screening.** (See color insert.)

4. Target and Pathway Identifications

The target identification of bioactive small molecules remains one of the major challenges in the field of molecular pharmacology. Various systemic approaches have been developed and used to facilitate target and mechanism determination, including affinity chromatography, genome-wide mRNA expression analysis, proteomic profiling by mass spectroscopy or protein array, and large-scale gene complementation assays using arrayed cDNA and siRNA libraries. Among these approaches, conventional affinity pull-down using small-molecule–immobilized solid matrixes is still the most straightforward and unbiased biochemical approach in target identification. However, new tricks have recently been implemented (Burdine and Kodadek, 2004), such as affinity linker optimization, blocking/soaking nonspecific binding sites in the matrix, reverse affinity chromatography (using a generic form of small-molecule–affinity resin to capture interested proteome and applying the specific small molecule to elute and compete with the compound targets in a dose-dependent manner), combinatorial chromatographic analysis with multiple optimized positive and negative affinity resins, and *in situ* affinity labeling.

DNA microarrays have been extensively used to generate global gene expression profiles in which differentially and temporally modulated gene clusters may reveal primary responding signaling pathways. Expression profiling in conjunction with systematic pathway analysis has been shown to provide useful information in small-molecule target and signaling identification, validation, and MOA studies. Additional emerging tools, such as proteomic profiling, add another dimension of information to dissecting the small molecules' MOA.

Furthermore, large-scale complementation assay using spatially addressable arrayed cDNA (Michiels et al., 2002; Carpenter and Sabatini, 2004; Huang et al., 2004) and small interfering RNA (siRNA) (Elbashir et al., 2001; Aza-Blanc et al., 2003; Zheng et al., 2004) libraries have been explored for the functional identification of proteins involved in the compound-targeted pathway. The fundamental notion of this technique is that the effect of a given bioactive molecule can be modulated (shift of the dose–response curve) by the overexpression or suppression of a particular set of genes that is involved in the signaling pathway, which the drug targets. Such an assay has been widely used in yeast and mammalian systems as described in the functional genomic approaches in the previous section.

Although one particular approach discussed previously may be more informative than others for revealing the target and pathway of a specific compound, information combined from different approaches may ultimately shed light on the MOA of the small molecule. Most importantly, genes,

signaling pathways, and other knowledge gained from these systematic analyses may serve as new entry points for additional investigations of the biologic phenomenon of interest.

B. Small-Molecule Regulators of Stem Cell Fate

1. Self-Renewal

Sustained stem cell self-renewal requires the combined forces of proliferation, the inhibition of differentiation, and the prevention of apoptosis. ESCs are conventionally maintained on feeder cells or in mixtures of exogenous factors. mESCs can be expanded in the pluripotent state in a defined medium supplemented with leukemia inhibitory factor (LIF) and bone morphogenetic protein (BMP), whereas the self-renewal of hESCs requires bFGF. Interestingly, 6-bromoindirubin-3'-oxime (BIO), a natural product derived from mollusk Tyrian purple, has been shown to maintain mESCs in the undifferentiated state in the conventional serum-containing media without feeder cells and LIF (Sato et al., 2004). BIO was proposed to function by inhibiting glycogen synthase kinase 3 (GSK3) and activating the canonical Wnt signaling pathway, although the precise mechanism and relevance of canonical Wnt signaling to ESC self-renewal remain to be determined. In mESCs, BMP functions by activating transcription factor Id through Smad. In addition, BMP inhibits both extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinases (MAPKs), which have been shown to be negative regulators for mESC self-renewal. Consistent with those findings, both MAPK/ERK kinase (MEK) inhibitor PD98059 (Burdon et al., 1999) and p38 inhibitor SB203580 (Qi et al., 2004) have positive effects on the promotion of the self-renewal of mESC. Recently, an imaging-based high-throughput chemical screen that combined both gene expression (Oct4, a pluripotency marker) and morphologic (undifferentiated ESCs grow as compact colonies) analyses was carried out in an Oct4-GFP reporter mESC line to identify small molecules that control the self-renewal of mESCs. A novel pyrimidine derivative, pluripotin, was discovered, and it is sufficient to propagate mESCs in the pluripotent state under chemically defined conditions in the absence of feeder cells, serum, and LIF. Long-term pluripotin-expanded mESCs can be differentiated into cells in the three primary germ layers *in vitro*, and they can also generate chimeric mice and contribute to the germ line *in vivo*. Interestingly, pluripotin does not operate through the known signaling pathways (i.e., LIF, BMP, and Wnt) that control the self-renewal of mESCs. Affinity chromatography using a pluripotin-immobilized matrix identified ERK1 and RasGAP as the molecular targets of pluripotin. Additional biochemical and genetic experiments suggest that pluripotin is a dual-function small-molecule inhibitor of both ERK1 and RasGAP and that the simultaneous inhibition of both protein activities is necessary and sufficient for pluripotin's effects on mESCs. ERK activation has been implicated in the differentiation of mESCs. Consequently, the inhibition of ERK1 by pluripotin would be expected to contribute to the self-renewal of mESCs. RasGAP modulates Ras signaling by stimulating the guanosine triphosphate (GTP)–hydrolysis activity of Ras to form the inactive RasGDP complex. By inhibiting RasGAP, pluripotin may activate signaling by Ras or Ras-like GTPases, which in turn may enhance self-renewal through phosphatidylinositol 3-kinase or other signaling pathways. In addition to serving as a useful chemical tool for the control of the self-renewal

of ESCs, pluripotin also represents a novel class of dual inhibitors of a protein kinase and a small GTPase activating protein (Chen et al. 2006). This discovery provided the first evidence that one small molecule is sufficient to sustain long-term self-renewal of mouse ESCs by modulating more than one signaling pathways. Traditionally, ESCs are maintained in self-renewal by mitogens and cytokines through activation of plasma membrane associated receptors. Since expression of stem cell specific genes is active at the specific developmental stage when ESCs are isolated and established in culture, we believe the activation of these pathways functions mostly to inhibit various differentiation potentials and balance cells in a multipotent self-renewal state. The balance is achieved by cross suppression of the lineage specific differentiation related genes. Our results indicated that the activation of these receptors is not absolutely required. Cell membrane permeable small molecules can bypass the upstream molecules and directly activate the downstream signaling, and thus maintain ESCs in a self-renewal state. Such discovery may provide new insights into the self-renewal mechanism and facilitate the practical applications of ESCs in research and therapy.

2. Lineage-Specific Differentiation

The most commonly used method for inducing the differentiation of ESCs involves growing them in suspension (in the presence of serum and the absence of supplemented LIF) to form aggregates called embryoid bodies (EBs), which begin to differentiate into various cell lineages, including hematopoietic, endothelial, neuronal, and cardiac muscle cells. However, such uncontrolled differentiation is a poorly defined, inefficient, and relatively nonselective process, and it therefore leads to heterogeneous populations of differentiated and undifferentiated cells, which are not useful for cell-base therapy and which also complicate biologic studies of a particular differentiation program. Consequently, dissecting stem cell signaling pathways and identifying critical factors that are involved in tissue specification are essential for the development of stem cell therapy and related small-molecule therapeutics. A number of small molecules have been identified that modulate specific differentiation pathways of embryonic or adult stem cells.

a. Neural and neuronal differentiation

RA is a widely used small molecule for the neural and neuronal differentiation of ESCs and neural cells. The effect of RA is dose and developmental stage dependent. It was recently demonstrated that subtype-specific neurons can be generated from mouse and human ESCs in a stepwise fashion. For example, to generate motor neurons, mESCs were first neuralized through EB formation with concomitant RA treatment. The generated neural cells were further caudalized by RA, and this was followed by treatment with a specific small molecule agonist (Hh-Ag1.3) of Sonic hedgehog signaling to ventralize the caudalized neural cells to become the desired motor neurons. This experiment suggests that multiple sequential signals, a combination of signals, or both may be required to generate a terminally differentiated, subtype-specific cell type. TWS119, a synthetic disubstituted pyrrolopyrimidine, was recently identified from a reporter-based screen as a potent inducer of neuronal differentiation in pluripotent mESCs and P19 murine embryonal carcinoma cells (Ding et al., 2003). A panel of affinity matrices prepared from

representative TWS analogs were used to pull-down target proteins from P19 cell extracts. Proteins specifically bound to all positive resins derived from active molecules but not to the negative resins derived from inactive molecules were considered to be the putative targets of TWS119. Consequently, GSK3β was identified as one target of TWS119 and confirmed by additional biochemical and cellular assays. This target identification may provide yet another link between neuronal differentiation and the Wnt signaling pathway. Additional studies also indicated that TWS119 (like BIO) is not entirely specific against GSK3β. Alternatively, TWS119 might promote neuronal differentiation of mESCs via novel mechanisms other than the canonical Wnt signaling pathway. Such mechanisms might include the compound's inhibition of other proteins that were not apparent in the affinity experiments (possibly as a result of low abundance or other factors) or cross-talk of the Wnt pathway with other signals present in the media.

Neuropathiazol (a substituted 4-aminothiazole) was recently identified from a high-content imaging-based screen of chemical libraries that specifically induces the neuronal differentiation of multipotent adult hippocampal neural progenitor cells. Treatment of the neural progenitor cells with neuropathiazol significantly slowed cell proliferation without visible cytotoxic effects; more than 90% of cells differentiated into neuronal cells as determined by immunostaining with βIII tubulin and the characteristic neuronal morphology (Warashina et al., 2006). In addition, reverse transcription polymerase chain reaction (RT-PCR) of marker genes showed that Sox2 (a neural progenitor marker) was downregulated and that NeuroD1 (a neuronal differentiation marker) was upregulated after treatment with neuropathiazol. Interestingly, neuropathiazol can also inhibit the astroglial differentiation induced by LIF and BMP2 whereas RA cannot, which suggests that neuropathiazol functions by a different mechanism and that it has more specific neurogenic-inducing activity than RA. In addition to the unbiased screening approach, modulating defined molecular targets that have been implicated in self-renewal and the differentiation of neural stem and progenitor cells by selective small molecules has provided a rationalized means of generating desired cell types in a controlled manner. For example, Hedgehog (Hh) pathway agonists were used to promote the proliferation of adult hippocampal neural progenitors, whereas histone deacetylase inhibitors were shown to specifically induce their differentiation into neurons.

b. Cardiomyogenic differentiation

The mammalian adult heart, like the brain, is mainly composed of postmitotic and terminally differentiated cells. Although there is evidence suggesting a resident population of self-renewing cardiac stem cells that is able to contribute to heart repair, the scarcity of these cells and their intrinsically poor regenerative response to heart injury remain obstacles for their therapeutic application. Alternatively, pluripotent ESCs represent a possible unlimited source of functional cardiomyocytes. A recent study has shown that hESC-derived cardiomyocytes can form structural and electromechanical connections with a primary culture of neonatal rat ventricular myocytes *in vitro* and pace the heart of pigs that had complete heart block *in vivo*, suggesting that hESC-derived cardiomyocytes may act as potential rate-responsive biologic pacemakers for myocardial repair (Kehat et al., 2004).

The cardiomyogenesis of ESCs can occur after EB differentiation *in vitro*, but such a condition is very inefficient and nonspecific. Consequently, the development of new approaches for the directed differentiation of ESCs into cardiomyocytes will facilitate the therapeutic applications of ESCs and increase our understanding of the molecular mechanism underlying cardiomyocyte differentiation and heart development. Using mESCs that are stably transfected with the cardiac-muscle–specific myosin heavy chain promoter-driven eGFP, ascorbic acid (vitamin C) was identified from a screen of known drugs that can enhance the cardiac differentiation of mESCs in the monolayer culture (Takahashi et al., 2003). Interestingly, other antioxidants (e.g., N-acetylcysteine, vitamin E) do not have a similar effect, which suggests that the cardiomyogenesis-inducing activity of ascorbic acid may be independent of its antioxidative property. A similar screening strategy has identified cardiogenol (a substituted diaminopyrimidine) from large combinatorial chemical libraries as a compound that can selectively and efficiently induce the differentiation of mESCs to cardiomyocytes in monolayer cultures (Wu et al., 2004a). The differentiated cells expressed multiple cardiac muscle markers, including GATA-4, Nkx2.5, MEF2, and myosin heavy chain, and the differentiated culture formed large areas of spontaneous contracting patches.

c. Differentiation of mesenchymal stem and progenitor cells

MSCs are multipotent cells with significant cellular plasticity. They can differentiate into a variety of mesenchymal tissues, such as osteocytes, adipocytes, and chondrocytes; and they can also differentiate into other tissue types, such as neuronal and skeletal muscle cells, under specific differentiation conditions. A number of small molecules have been found that can be used to control the differentiation of mesenchymal stem or progenitor cells for a variety of applications. For example, 5-aza-C (a DNA demethylation chemical) can induce C3H10T1/2 cells (a mouse mesenchymal progenitor cell line) to differentiate into myoblasts, osteoblasts, adipocytes, and chondrocytes. 5-Aza-C does not directly activate a specific differentiation program, but rather it converts the cells into a competent differentiation state. Dexamethasone (a glucocorticoid receptor agonist), ascorbic acid, β-glycerophosphate, isobutylmethylxanthine (a nonspecific phosphodiesterase inhibitor), and peroxisome proliferator-activated receptor γ gonists (e.g., rosiglitazone) have been widely used to modulate the osteogenesis or adipogenesis of MSCs under specific conditions. Interestingly, treatment with a JAK inhibitor (WHI-P131) followed by trophic factor induction was recently shown to be able to convert rat MSCs into neuronal cells (Dezawa et al., 2004). Purmorphamine, a 2,6,9-trisubstituted purine compound, was identified as a potent osteogenic-differentiation–inducing molecule through a high-throughput chemical screen in C3H10T1/2 cells (Wichterle et al., 2002). Genome-wide expression profiling in conjunction with systematic pathway analysis was used to reveal that the Hh signaling pathway is the primary affected biologic network and that purmorphamine is a selective Hh pathway agonist. This was further confirmed by chemical epistasis using two different Hh pathway antagonists: cyclopamine, which binds and inhibits Smoothened (Smo), and forskolin, which activates protein kinase A to convert Gli proteins to transcriptional repressors by phosphorylation (Wu et al., 2004b). Additional biochemical assays have indicated that purmorphamine targets Smo (Sinha and Chen, 2006).

3. Proliferation Reactivation and Dedifferentiation

Terminally differentiated postmitotic mammalian cells are thought to have little or no regenerative capacity, because they are already committed to their final specialized form and function, and they have permanently exited the cell cycle. Their inability to regenerate (i.e., to divide and replace damaged tissue) may constitute a biomedical problem. Consequently, the stimulation of adult postmitotic cells to reenter the cell cycle and proliferate may provide new therapeutic approaches for treating degenerative diseases and injuries.

Mammalian cardiomyocytes remain proliferative during fetal development. Shortly after birth, the cell-cycle–perpetuating machinery shuts down, and cardiomyocytes lose their proliferative capacity. p38 MAPK was identified as a key negative regulator of mammalian cardiomyocyte proliferation through the regulating genes required for mitosis (including cyclin A and cyclin B). Recently, it was reported that a p38 inhibitor, SB203580, increased the growth-factor–induced S-phase progression and mitosis in both neonatal and adult cardiomyocytes indicated by BrdU incorporation and histone 3 phosphorylation. The proliferation in adult cardiomyocytes was also associated with the transient dedifferentiation of the contractile apparatus (Engel et al., 2005).

A longstanding notion in developmental biology has been that organ- and tissue-specific stem cells are restricted to differentiating into cell types of the tissue in which they reside. However, recent studies suggest that tissue-specific stem or progenitor cells may overcome their intrinsic lineage restriction after exposure to a specific set of signals *in vitro* and *in vivo*, although such reprogramming may not reflect potentials that are normally exercised *in vivo*. An extreme example is the reprogramming of a somatic cell to a totipotent state by nuclear transfer during which the nucleus of a somatic cell is transferred into an enucleated oocyte. The ability to dedifferentiate or reverse lineage-committed cells back to multipotent or even pluripotent cells might overcome many of the obstacles associated with using ESCs and nonautologous stem cells in clinical applications. However, the cellular processes involved in dedifferentiation remain poorly understood, and methods for the control and study of dedifferentiation are lacking.

To identify small molecules that can induce the dedifferentiation of C2C12 myoblasts, an assay was designed based on the notion that lineage-reversed myoblasts should regain multipotency, which is the ability to differentiate into multiple mesenchymal cell lineages under conditions that typically only induce the differentiation of multipotent MSCs into adipocytes, osteoblasts, or chondrocytes. Reversine, a 2,6-disubstituted purine, was found to have the desired dedifferentiation inducing activity: it inhibits the myotube formation of C2C12 myoblasts, and only reversine-treated myoblasts can efficiently differentiate into osteoblasts and adipocytes after exposure to the appropriate differentiation conditions. Importantly, the efficient dedifferentiation effect of reversine on C2C12 cells can be shown at the clonal level, which suggests that this effect is inductive rather than selective. Furthermore, reversine appears to have similar effects on several other primary and established cell lines, which suggests that its mechanism may be general in cellular reprogramming. Affinity chromatography and other cellular studies revealed that the mechanism of reversine's action is twofold: 1) to stage cells at a specific phase in cell cycle by interfering with a cell cycle regulator; and 2) to enhance

cytoskeletal rearrangement and growth-factor–induced reprogramming by targeting proteins involved in both pathways. This example is a proof-of-principle demonstration that the dedifferentiation of lineage-restricted cells to a more primitive (multipotent) state by a synthetic chemical can be achieved via a rationally designed phenotypic screen of combinatorial chemical libraries and that such concepts and technologies are readily applicable to other models.

C. Regeneration Screens

Regeneration screens have been carried out at both the cellular and whole-organismal levels to identify the small molecules and genes involved in the regenerative process. Inhibitory molecules associated with myelin and the glial scar limit axon regeneration in the adult CNS, but the underlying mechanism of such regeneration inhibition is not fully understood. A small molecule screen to search for compounds that can neutralize neurite outgrowth inhibitory activity associated with the CNS myelin identified several epidermal growth factor receptor kinase inhibitors. These compounds showed a remarkable ability to counteract the effects of myelin inhibition, and they promoted the significant axon regeneration of injured optic nerve fibers, which points to a promising therapeutic avenue for enhancing axon regeneration after CNS injury (Koprivica et al., 2005).

In a recent study that examined zebrafish fin regeneration, disorganized mesenchymal cells beneath the amputation plane were observed, and this was followed by cell proliferation, migration, and blastema formation, which provided evidence of dedifferentiation. The blastema is a mass of undifferentiated mesenchymal cells that have proliferated beyond the amputation plane to drive fin regrowth. In a screen of the *N*-ethyl-*N*-nitrosourea–induced mutants that fail in fin regeneration, fgf20a null mutants were identified, which indicates that fgf20a is required for regeneration-specific blastema formation and subsequent fin regeneration (Makino et al., 2005).

Osteoporosis and diseases of bone loss are a major health problem associated with aging. The bisphosphonates have been widely used for the treatment of osteoporosis by inhibiting bone absorption. However, there are no agents that promote bone formation. To facilitate the identification of novel anabolic molecules, a high-throughput *in vivo* screen using larval zebrafish has been performed in a 6-day time period. Vitamin D3 analogs and intermittent parathyroid hormone were shown to cause dose-dependent increases in the formation of mineralized bone (Fleming et al., 2005). This fast, economical, and genetically tractable screening system provides a powerful adjunct to mammalian models for the identification of bone anabolic agents, and it offers the potential for the genetic elucidation of the pathways involved in osteoblastic activity.

D. Chemical Screens of Pathways

Fundamental developmental signaling pathways (e.g., Wnt, Hh, BMP, Notch; see Chapter 1), which control embryonic patterning and cell behaviors, play important roles in stem cell regulation. The deregulation of these pathways in either the embryonic or adult stage may result in diseases, such as cancer and degenerative disease.

In addition to compounds that were discovered via other means (e.g., purmorphamine and cyclopamine-Hh pathway agonist and antagonist, respectively; BIO and sulindac-Wnt pathway agonist and antagonist, respectively; DAPT-Notch pathway antagonist), cell-based pathway-specific screens have also been used to identify the small-molecule regulators of these developmental pathways. For example, a series of Hh-pathway–specific agonists and antagonists have been identified through screens of synthetic compounds using 10T1/2 cells stably transfected with a plasmid containing a luciferase reporter downstream of multimerized Gli binding sites and a minimal promoter. These molecules have been used in various applications ranging from stem cell proliferation and differentiation to the induction of apoptosis in cancer cells. By carrying out Wnt pathway-specific screens of a chemical library using the TOPflash reporter assay, a 2-amino-4,6-disubstituted pyrimidine compound was identified that activates Wnt signaling in a dose-dependent manner. Interestingly, this compound does not inhibit GSK-3β, which is a major inhibitory component in the pathway; however, its activity could be blocked by a dominant negative T cell factor 4 (TCF4), which suggests that it functions upstream of the known TCF factors on the canonical Wnt signaling pathway. Importantly, this compound appears to mimic the effects of Wnt ligand in a *Xenopus* model, which suggests that it may be a useful tool to study physiologic processes that involve Wnt signaling. From a Wnt3a sensitized screen, a 2,6,9-trisubstituted purine compound was identified as a Wnt synergistic agonist. Consistent with its *in vitro* activity, this purine compound—in combination with a suboptimal dose of XWnt8—induces axis duplication in *Xenopus,* with high penetrance. Affinity chromatography study in conjunction with genetic confirmations has identified a GTPase activating protein of ADP-ribosylation factor (ARF-GAP) as a target of this purine compound. ARF family GTPases are best known for their function in vesicle transport. By inhibiting ARF-GAP, this purine compound activates ARF, which leads to increased levels of the cytoplasmic β-catenin; however, only in the presence of Wnt pathway activation is such translocated β-catenin stabilized to further activate downstream transcription. This mechanism may provide one explanation for the function of this purine compound as a synergistic agonist of Wnt signaling.

IV. CONCLUDING REMARKS

Stem cell biology is a fast-growing field that offers new opportunities for the treatment of many devastating diseases and that provides new insights into the molecular mechanisms that control developmental processes. However, there remain significant obstacles that must be overcome before the therapeutic potential of stem cells can be realized. This requires a better understanding of the signaling pathways that control stem cell fate and an improved ability to manipulate stem cell proliferation and differentiation. Functional genomic and proteomic studies at the systems level are very likely to yield insights into the fundamental molecular mechanisms underlying stem cell fate determination.

Although the small-molecule approach has been practiced in drug discovery and used in probing biology for decades, its value in the stem cell field is just now beginning to be realized (Figure 3.3). However, many challenges

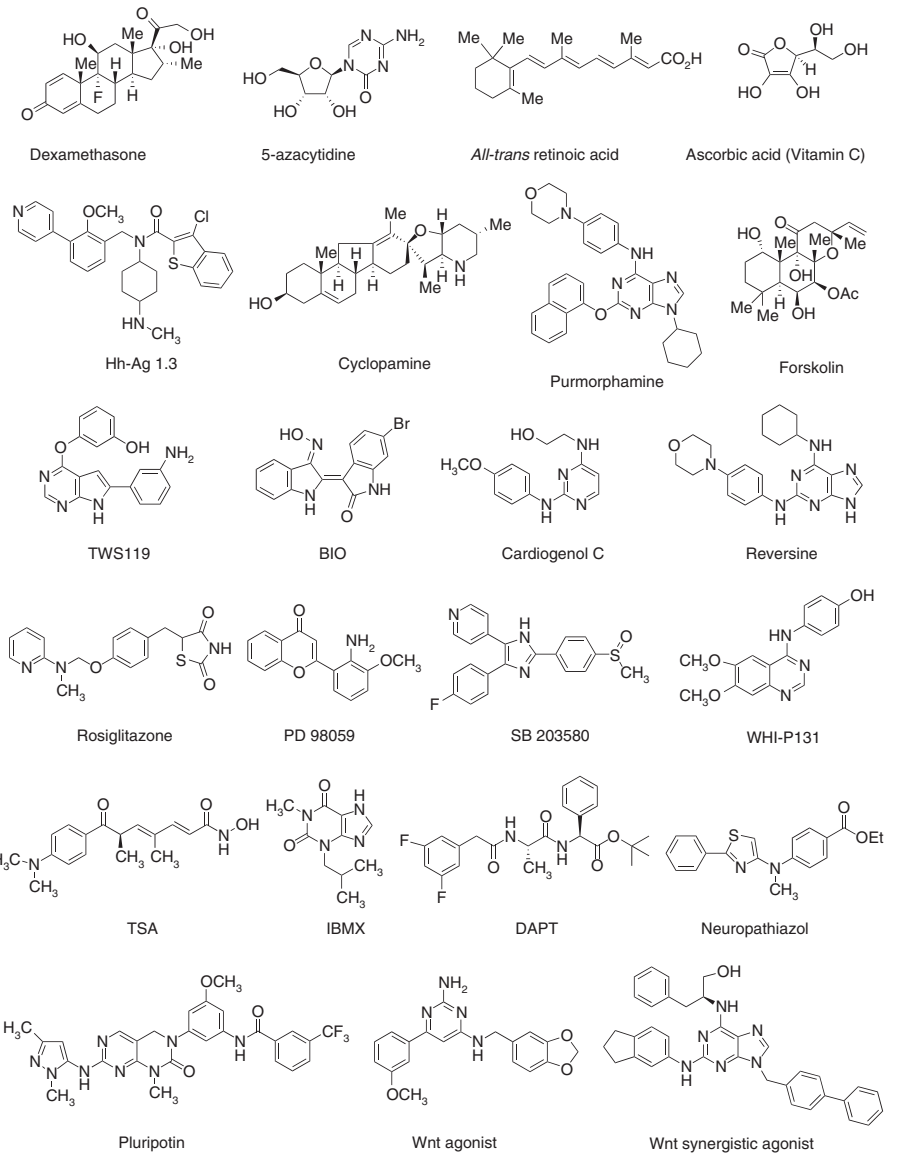

FIGURE 3.3 **Small compounds modulating stem cell fate.**

remain, including designing better chemical libraries and screening strategies to systematically identify the small molecules that regulate the desired cellular process, developing more efficient methods to understand the underlying mechanisms, and translating *in vitro* discoveries into approaches for the *in vivo* regeneration of desired tissues and organs by small-molecule therapeutics. Nonetheless, it is clear that the identification of additional small molecules that control stem cell fate will significantly facilitate the studies of stem cell and developmental biology and contribute to the development of regenerative medicine.

SUMMARY

- Embryonic stem cells are pluripotent cells that have the potential to differentiate into essentially all cell types in the organism, whereas tissue-specific adult stem cells are multipotent with the restricted potential to differentiate into certain specific cell types.
- It remains challenging to maintain stem cell self-renewal or to direct lineage-specific differentiation in a homogenous fashion. Methods for the precise control of stem cell fate will not only allow for the generation of desirable cells for cell-based therapy, but they will also provide excellent systems for studying the underlying mechanisms that control such processes.
- The realization of stem cells' therapeutic potential will require an improved ability to control their fate and a better understanding of the precise molecular mechanisms underlying their proliferation, differentiation, migration, and survival at the systems level.
- The application of chemical and genomic approaches in stem cells will greatly advance our understanding of fundamental questions in stem cell and developmental biology, and it may ultimately facilitate the development of novel therapeutic strategies to treat human diseases and to stimulate tissue and organ regeneration *in vivo*.

GLOSSARY OF TERMS

Functional genomics
Functions of a genome-scale collection of genes are simultaneously evaluated in a biologic process.

High-throughput screening
Recent advances in automation and detection technologies allow for the evaluation of a large number of genes or small molecules at the same time in a certain biologic process.

MOA
Mechanism of action studies of a small molecule, including target and/or pathway identification by various methods.

Proteomics
Protein profiling technologies (e.g., mass spectrometry) for examining protein–protein interactions and/or protein modification and activities.

Structure-and-activity-relationship studies
Studies of a small molecule involving reiterated rounds of testing structurally related compounds modified via medicinal chemistry around substituent and scaffold.

Self-renewal
The symmetric or asymmetric division of a stem cell into two identical or different daughter cells (with one identical to the parental cell) to replicate itself and maintain the potential for differentiation. It requires combined forces of promotion of proliferation, inhibition of differentiation, and prevention of apoptosis.

SILAC
Stable isotope labeling by amino acids in cell culture. It is a simple and cost-effective approach to quantitative proteomics.

Stem cells
Cells that have the ability to self-renew for long periods of time and to differentiate into specialized cell types in response to appropriate signals, including pluripotent embryonic stem cells and multipotent adult (tissue-specific) stem cells.

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FURTHER READING

http://stemcells.nih.gov/stemcell/scireport.asp

Stanford Genomic Resources
http://genome-www.stanford.edu/

Science, Functional Genomics Home
http://www.sciencemag.org/feature/plus/sfg/

FunctionalGenomics.org.uk
http://www.functionalgenomics.org.uk/

Functional and Comparative Genomics: Human Genome Research in Progress
http://www.ornl.gov/sci/techresources/Human_Genome/research/function.shtml

Harvard Institute of Proteomics
http://www.hip.harvard.edu/

4

ASSESSING NEURAL STEM CELL PROPERTIES USING LARGE-SCALE GENOMIC ANALYSIS

SOOJUNG SHIN, JONATHAN D. CHESNUT, and MAHENDRA S. RAO

Invitrogen Corporation, Carlsbad, CA

INTRODUCTION

The nervous system is one of the earliest organ systems to differentiate from the blastula stage embryo. Neural stem cells (NSCs) are the cells in the nervous system that give rise to all neurons and supporting glial cells by symmetric and asymmetric divisions. This can be mimicked in culture, and NSCs can be derived from human embryonic stem cell (ESC) cultures over a period of 2 to 3 weeks (Reubinoff et al., 2001; Shin et al., 2006; Zhang et al., 2001). ESCs are the *in vitro* counterpart of the inner cell mass of the blastula-stage embryo, which gives rise to every component of our body. *In vivo*, the primitive neural tube forms by approximately the fourth week of gestation, and neurogenesis has commenced by the fifth week of development in humans (Kennea et al., 2002). At the time of neurulation or around the fourth week (see Chapter 12), the neuroectoderm segregates from the ectoderm by a process called *neural induction*. The initially formed neural plate then undergoes a stereotypic set of morphogenetic movements to form a hollow tube, which is comprised primarily of stem cells by a process called *primary neurulation* (Rao, 1999). The neural crest, which will form the peripheral nervous system, segregates from the central nervous system at this stage (see Chapter 26). Stem cells that will generate the central nervous system reside in the ventricular zone (VZ) throughout the rostrocaudal axis; they appear to be regionally specified; they proliferate at different rates; and they express different positional markers. The anterior neural tube undergoes a dramatic expansion and can be delineated into three primary vesicles: the forebrain (prosencephalon), the midbrain (mesencephalon), and the hindbrain (rhombencephalon). Differential growth and further segregation lead to the additional delineation of the prosencephalon into the telencephalon and the diencephalon and of the rhombencephalon into the metencephalon and the myelencephalon. The caudal neural tube does not undergo a similar expansion, but it does increase in size to parallel the growth of the embryo, and it undergoes further differentiation to form the spinal cord. The properties of VZ stem cells have been characterized (Rao, 2004; Schubert et al., 2000), and they appear to be homogenous, despite the acquisition of rostrocaudal and dorsoventral identity.

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As development proceeds, the VZ is much reduced in size, and additional zones of mitotically active precursors can be identified. Mitotically active cells that accumulate adjacent to the VZ have been called *subventricular zone (SVZ) cells*. This SVZ is later called the *subependymal zone* as the VZ is reduced to a single layer of ependymal cells. The SVZ is prominent in the forebrain, and it can be identified as far back as the fourth ventricle. No SVZ is detectable in the more caudal regions of the brain, and, if it exists, it is likely a very small population of cells. An additional germinal matrix that is derived from the rhombic lip of the fourth ventricle, called the *external granule layer,* generates the granule cells of the cerebellum. Like the VZ, the SVZ can be divided into subdomains that express different rostrocaudal markers and that generate phenotypically distinct progeny. Distinct SVZ domains identified include the cortical SVZ, the medial ganglion eminence, and the lateral ganglion eminence. The proportion of SVZ stem cells declines with development, and, in the adult, multipotent stem cells are likely present only in regions of ongoing neurogenesis (e.g., the anterior SVZ, the SVZ underlying the hippocampus). At this stage, marker expression is relatively heterogeneous (Bernier et al., 2000; Doetsch et al., 1996; Pevny et al., 2003).

Stem cells do not generate differentiated progeny directly, but rather they generate dividing populations of more restricted precursors that are analogous to the blast cells or restricted progenitors described in the hematopoietic lineages (Bedi et al., 1995; Katsura et al., 2001; Mujtaba et al., 1999; see Chapter 34). These precursors can divide and self-renew, but they are located in regions that are distinct from the stem cell population, and they can be distinguished from the stem cell population by the expression of cell surface and cytoplasmic markers (Cai et al., 2004b; Kalyani et al., 1997; Liu et al., 2004). Investigators have begun efforts to analyze stem cell populations (Table 4.1) using a variety of techniques with the idea that, by understanding

TABLE 4.1 Methods That Have Been Used to Characterize Neural Stem Cell Populations

Authors	Cells Characterized	Method Used	Reference
Luo et al.	Neural stem cells and progenitor cells	Microarray	(Luo et al., 2002)
Cai et al.	Neuroepithelial cells	Subtractive suppression hybridization	(Cai et al., 2004b)
Liu et al.	Astrocyte-restricted precursors	Immunohistochemistry	(Liu et al., 2004)
Svendsen	Neural stem cells	Microarray	(Wright et al., 2003)
Abramova et al.	SSEA1-positive cells	Microarray	(Abramova et al., 2005)
Geschwind et al.	Central nervous system progenitors	Microarray	(Geschwind et al., 2001)
Cai et al.	Neurosphere forming cells	Massively parallel signature sequencing	(Cai et al., 2006)
Miura et al.	Embryonic stem cells	Massively parallel signature sequencing	(Miura et al., 2004a)
Brandenberger et al.	Embryonic stem cells	Expressed sequence tag scan	(Brandenberger et al., 2004)
Richards et al.	Embryonic stem cells	Serial analysis of gene expression	(Richards et al., 2006)

these populations and identifying the factors that regulate self-renewal and direct differentiation, one will be able to modulate the development and response of stem cells to environmental signals. Many of these approaches depend on large-scale analytic tools that rely on the comparison of purified populations of cells that differ with regard to their stage of development or their exposure to factors or that carry specific genetic abnormalities. In this chapter, we focus on general principles that should guide such an analysis, the techniques used to perform such an analysis, and how data mining efforts have provided important insights into the properties of NSCs.

I. THE IMPORTANCE OF A GLOBAL ANALYSIS AND THE CAVEATS WHEN COMPARING CELL SAMPLES

The overall disposition of a cell depends on the steady state of a complex of interacting factors. The integration of these instructions occurs in the nucleus through combinations of signal-activated and tissue-restricted transcription factors binding to and controlling related enhancers or *cis*-regulatory modules of coexpressed genes. Additional regulation is provided by previously unappreciated epigenetic mechanisms such as histone modulation and CpG island methylation and by small untranslated RNA (microRNA). Thus, the response of the cell to any one perturbation is dependent on context, and it explains in part the conflicting results that have been reported. For example, the effect of Sonic hedgehog on NSCs is dependent on the presence or absence of fibroblast growth factor (Wechsler-Reya et al., 1999). Likewise, the response to bone morphogenetic protein depends on the density of the culture and the presence or absence of various regulatory genes (Rajan et al., 2003; Wilson et al., 2001). This context-dependent response suggests that the overall state of a cell needs to be understood before perturbation experiments are initiated so that consistent and meaningful analyses of the results can be obtained.

Several variables remain poorly understood. For example, no distinction has been made between long-term self-renewing populations and short-term self-renewing populations. Although the evidence that stem cells age is quite clear (Shen et al., 1998; Svendsen, 2000), no analysis so far has taken into account the effects of aging, the acquisition of karyotypic abnormalities, the differences as a result of the acquisition of positional identity, or the differences between types of NSCs that are present during development. For example, radial glia type stem cells, transdifferentiated stem cell populations, VZ stem cells, SVZ-derived stem cells, and neurosphere-forming stem cells fulfill the criteria of NSC such as self-renewal and the ability to differentiate into neurons and glia, but the comparisons among them have not been fully understood.

Two other observations have suggested that caution needs to be exercised as stem cell populations are analyzed. Stem cells propagated in culture stochastically differentiate, and, as such, they are invariably contaminated by various amounts of differentiated cells. For example, the proportion of stem cells in a neurosphere culture can vary from 1% to 2% to up to 100%. Notably, the largest contaminating populations are astrocytes and astrocyte precursors, which are dividing and expressing Nestin in culture. A confounding point is that these cells are difficult to distinguish from stem cells using the

TABLE 4.2 Advantages and Disadvantages of Various Methods of Gene Expression Analysis

	Massively Parallel Signature Sequencing	Serial Analysis of Gene Expression	Microarray
Minimum amount of sample required	20 μg of total RNA	~1 μg of total RNA	~1 μg of total RNA
Detection capacity	~2,000,000	~100,000	~48,000
Data presentation	Transcripts per million	Tag number detected	Hybridization intensity using fluorescence
Sensitivity	+++++	+++	++
Data calibration and standardization	Not required	Not required	Required
Technical biases	Failure of sequencing reaction; duplication of the sequence	Failure of sequencing reaction; duplication of the sequence	Background hybridization from rivaling signal; signal overlapping; detector saturation
Cost	High	High	Low
Turnaround time	Long (10 weeks)	Long (6 months)	Short

standard battery of tests. A second important observation made was that there are species differences between stem cells, and, thus, extrapolating from mouse to human is fraught with caveats (Barker et al., 2003; Ginis et al., 2004). The genome data sets for the two species are also not identical, thereby making cross-species comparisons difficult to interpret. Each of these differences will add variability to the results and make cross-laboratory comparisons difficult unless attention is paid to the quality of the sample and detailed information is provided regarding the time of isolation, the age at which the cells are isolated, the number of passages in culture, and the degree of contaminating cells present. These differences must be documented and taken into account when comparing data sets, because the noise from such variability can mask important critical differences between cell populations. In addition, when analyzing the cells, it is desirable to use a reliable and reproducible method that is cost-effective and sensitive enough to detect with high fidelity the global differences among populations of cells as well as the subtle differences introduced as the cells are propagated in the culture or as they mature. Although several different methodologies have been proposed, none of the cross-platform comparisons is very useful unless sophisticated normalizing algorithms and consideration of the technical variables inherent in large-scale analyses are carefully considered (Table 4.2).

Nevertheless, as improvements are made in the ability to obtain pure populations of cells, to harvest RNA from single cells or small amounts of tissue, to construct libraries, to sort cells, and to obtain high-quality genomic information, such large-scale analysis has become increasingly possible. However, it is recommended that analysis and comparisons be limited to one stage of development, in one species, with a single platform. The samples should be carefully examined for the presence of contaminating populations, and the degree of contamination should be assessed (Figure 4.1). This initial quality control will be critical for the yielding of useful results.

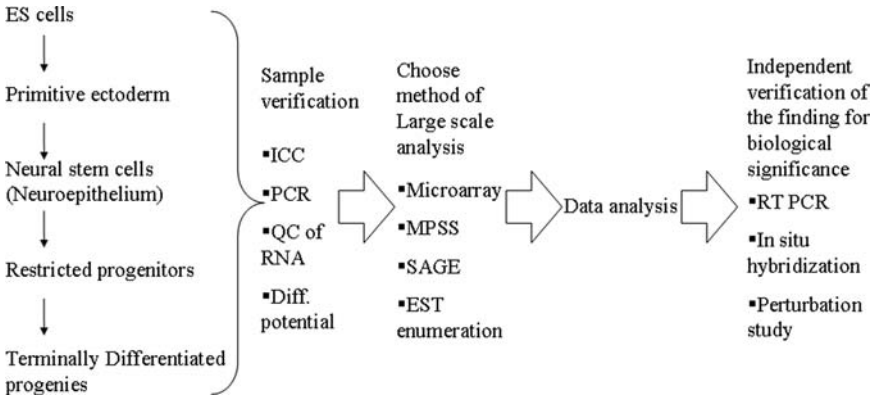

FIGURE 4.1 Flowchart of techniques used to characterize cell populations using large-scale analysis. Samples for global analysis require verification in advance to ensure dependable data production. After the quality of the sample is controlled, the method of large-scale analysis that is most appropriate for the purpose needs to be determined. After the generated data set is processed and analyzed, an independent method is selected to confirm the acquired results.

II. THE USE OF A REFERENCE STANDARD

When comparing large batches of data generated in different laboratories using different techniques or slightly different cell culture protocols, one must determine how best this can be done. Several strategies have been proposed, including the idea of a reference standard (Dybkaer et al., 2004; Novoradovskaya et al., 2004). This idea, although new, appears to be underappreciated in the stem cell field. However, most researchers have found it all but impossible to mine across data sets, because there are too many variables that need to be normalized and too many assumptions that need to be made. Furthermore, there are often circumstances in which one simply lacks the data to make any appropriate assumptions. In the ESC field, several strategies have been proposed (Loring et al., 2006). These include establishing a publicly available and well-curated data set that can be used as a ready reference, a set of standards that are readily available from a commercial or not-for-profit provider, or a control sample that all investigators can use as a standard. In principle, each of these could be applied to the NSC field, but, to our knowledge, no such common database exists as yet.

Immortalized or cancer stem cell lines, such as C17.2, RT-4 or more recently identified cancer stem cell lines harvested from the appropriate species of interest, have been proposed as possible standards (Imada et al., 1978; Snyder et al., 1992; Steindler, 2002). However, it is important when using such lines as a reference to carefully assess the subclone that is being used. C17 subclones, for example, have shown remarkable variability, and diametrically opposite results have been reported, depending on the subclone used. The karyotype of this line is unstable, which may account for some of the differences seen. Nevertheless, because it has been so widely used, it could serve as a reference, provided that sufficient care was taken to use the same passage sample banked at American Type Culture Collection or some other responsible cell-banking facility.

Fetal tissue samples from which pure populations of stem cells can be harvested at a defined stage of development in rodents may be an alternative choice for a reference standard. Many commercial entities provide such

samples, and these could therefore become a *de facto* standard. The equivalent stages of development are not readily accessible in humans, however, and, as such, an alternative control will need to be considered. Sorting or negative-selection strategies have been shown to enrich for stem cell populations, and markers that define the stem cell stage are available; thus, a reference standard for human stem cell analysis could be considered. Alternatively, a publicly available and well-curated data set could be provided that could serve as a digital reference standard.

In the absence of any of the above, we recommend obtaining RNA, genomic DNA, and microRNA from NTERA2. This is not an NSC line, but it readily differentiates into neurons and glia. It has been carefully analyzed by several groups, and labeled and unlabeled clones are both commercially available and available from American Type Culture Collection. The line is often used as a comparator for ESC work, and, thus, significant data on several different platforms are already available. However, these cells are not optimal when detailed high-resolution comparisons are required (Schwartz et al., 2005).

In our laboratory, having an internal reference standard has proven invaluable in allowing us to compare NSCs to each other and to samples run at different times and to compare our results with those of several other colleagues without the necessity of repeating all of the experiments. These standards have also allowed us to check the quality of markers, our fluorescence activated cell sorting efficiencies, and the quality of our antibodies, and they have provided a basis for comparing across different laboratories. By running a reference sample in one laboratory and sending results to another laboratory, our experiments can be easily compared, and, over time, cross-platform comparisons also become possible (Figure 4.2).

After the concept of a standard is accepted widely, commercial providers can provide RNA, DNA, and genomic material from such a reference that can

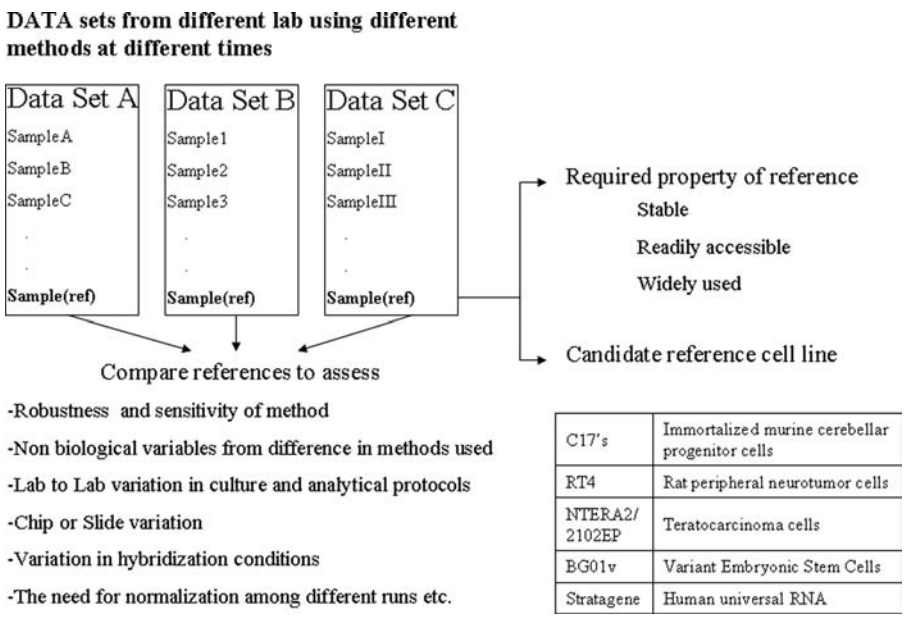

FIGURE 4.2 **The importance of using a reference standard.** The reference standard makes it possible to compare data sets from different slides, different time points, different laboratories, and even different methods.

be used as a comparator for all types of large-scale studies. In the ESC and microarray fields, groups have been established to determine such standards, and such uniformity has yielded useful results (Brazma et al., 2001; Husser et al., 2006; Wei et al., 2005), and we fully expect that a similar effort in the NSC field will be equally useful.

III. METHODS OF ANALYSIS

The past several years have seen dramatic advances in technology and equally dramatic reductions in cost. Both genomic and proteomic methods are now available at costs that allow an average small laboratory to begin performing such experiments. The amount of material required for such an analysis has also become much less than was previously necessary, thereby making these experimental approaches feasible even when the number of stem cells available is limited. For example, single-nucleotide polymorphism analysis to examine overall allelic variability using a 500-K chip set costs about $500. Likewise, a genome-wide gene expression profile using an Illumina 48,000 transcript chip costs about the same amount. Mitochondrial analysis and nuclear run-on assay expenses are also in the same ballpark. Even more importantly, the requirement of material has been dramatically reduced. We estimate that one can perform the entire battery of tests (excluding proteomic analysis) with about 2 million stem cells in any species. Approximately 10 million cells are enough for a mass spectrometry–based analysis, with equivalent amounts of material required for stable isotopic labeling using amino acids in cell culture and other similar methods. The material required will be further reduced as technology advances, and the costs are likely to be driven down further. Indeed, it is expected that whole-genome sequencing will cost less than $1000 in the near future and that profiling services will require one tenth of the current material. These and other technical advances have allowed large-scale gene expression analysis to be performed by a variety of techniques (Table 4.3), although many of the results remain unpublished.

In general, however, investigators have focused on gene expression profiling followed by epigenetic analysis and chromatin immunoprecipitation (ChIP)-on-ChIP type studies. Mitochondrial sequencing, histone and chromatin modifications, and run-on assays have not been used as frequently. In most cases, data have been limited to mouse and human cells, because the genomic databases that are required for such analysis have not been as well developed in other species. In the next section, we briefly describe some of the methods used.

A. Epigenetic Modulation

Over the past few years, the importance of heritable epigenetic remodeling has been highlighted in the regulation of stem cell proliferation, cell fate determination,

TABLE 4.3 Various Methods Used to Profile Cell Populations

Epigenetic modulation
MicroRNAs
Mitochondrial sequencing
Transcriptome mapping
Nuclear run-on assays
Proteomic analysis, glycosylation maps, and other protein-mapping strategies

and carcinogenesis (Beaujean et al., 2004; Huntriss et al., 2004; Meehan, 2003; Ohgane et al., 2004; Vignon et al., 2002). However, it has been difficult to study these events on a global scale. The ability to grow large numbers of cells and to differentiate them along specific pathways, coupled with the ability to perform such studies in a high-throughput fashion, suggests that this will change in the near future. Global methylation studies can be performed using a microarray (Maitra et al., 2005). Illumina has recently described a bead-array strategy to look at methylation patterns at 1500 loci encompassing regulatory elements in almost 400 genes. These include most genes known to be regulated during early embryonic development and those altered during tumorigenesis. These arrays have been used to examine methylation profiles in cancer stem cells and ESCs (Bibikova et al., 2006), and experiments assessing NSCs are underway. Assessing the epigenetic profile of NSCs will be important before using them for transplant therapy, because the maintenance of a particular epigenetic profile is probably critical for the appropriate function of cells.

B. MicroRNA

MicroRNAs are small noncoding RNA genes found in most eukaryotic genomes, and they are involved in the posttranscriptional regulation of gene expression. The microRNAs are transcribed in the cell nucleus, where they are processed into pre-microRNAs. Further processing occurs in the cytoplasm, where the pre-microRNAs are cleaved into their final ~22-nucleotide–long form, which appears to regulate gene expression via transcriptional, translational, or protein degradation regulation (Bartel, 2004; Szymanski et al., 2003). Recent reports have identified global strategies for identifying microRNAs, and more than 450 such untranslated RNAs have been identified in humans, mice, and other species (Houbaviy et al., 2003; Lewis et al., 2003; Rajewsky et al., 2004). These approaches include computational analysis using sophisticated algorithms that recognize potential microRNA coding sequences and potential binding sites. Other strategies have included making microRNA chips (Krichevsky et al., 2003) and sequencing protocols analogous to the massively parallel signature sequencing (MPSS) developed by Lynx therapeutics, which can be used to obtain quantitative data about microRNA made by a particular cell, which in turn predicts the overall state of the stem cell.

C. Mitochondrial Sequencing

Structural and functional abnormalities in mitochondria lead to functional defects in the nervous system, the muscles, and other organ systems. Somatic mitochondrial mutations are common in human cancers, aging cells, and cells maintained in culture for prolonged periods. Mitochondrial DNA is also relevant to nuclear transfer, and estimating the stability of it is important for assessing the response of cells to stress and for determining their ability to propagate in culture.

Techniques to examine mitochondrial DNA mutations have been under development for some time. A recent description of a polymerase chain reaction (PCR)-based approach for sequencing vertebrate mitochondrial genomes has attracted much attention for being more rapid and economical than traditional methods, which use cloned mitochondrial DNA and primer walking. Maitra et al. (2004) have developed a mitochondrial Custom RefSeq microarray as an array-based sequencing platform for the rapid and high-throughput

analysis of mitochondrial DNA. The MitoChip contains oligonucleotide probes synthesized using standard photolithography and solid-phase synthesis, and it is able to sequence more than 29 kb of double-stranded DNA in a single assay.

It is useful to note that many mutations arise in the D-loop regions and that a simple PCR amplification and sequencing process would capture a large amount of information. No published data about baseline mitochondrial sequence and its change after culture of NSC are currently available. However, several laboratories have initiated such experiments, and we expect data about the long-term viability of NSCs (insofar as mitochondrial stability) to be available soon.

D. Transcriptome Mapping

Efforts have begun to identify the complete DNA binding sites and corresponding genes targeted by the transcriptional factors. One approach is to use an *in silico* computational strategy to retrieve putative genes with such binding sites. A direct and physiologic approach is to perform the ChIP of factors cross-linked *in vivo* to DNA targets followed by the identification of the specific DNA binding sites. Promoter chips, which range from a focused selection of genes to complete gene sets, are now being made. The findings from such a project will be of immense biologic value in providing a description and understanding of the hierarchal relationships between groups of transcription factors and their target genes as they perform their tasks in embryonic development and the specification of lineage fates and terminal differentiation.

E. Nuclear Run-On Assays

One analogous approach to identifying regulatory elements is to perform the labeling of newly processed RNA to examine genes that are induced only after a specific stimulus. Such hybridizations, although they require larger amounts of material, are feasible with cell lines and with ESCs, and they can provide a global overview of the network of the transcriptional responses to a specific stimulus. More importantly, they provide an element of temporal control by allowing one to better place individual genes within a transcriptional network. Although such arrays have not been run with NSCs, experiments in other systems have yielded exciting results (Li et al., 2006), and we expect similar results from NSCs in the near future.

F. Proteomic Analysis, Glycosylation Maps, and Other Protein Mapping Strategies

Most of our discussion has been about methods for assessing genomic differences between cells. However, posttranslational modifications play a crucial role in modifying genomic information and increasing the complexity of information that can be processed by a cell. The very complexity of the proteome has made it difficult to study on a large scale. Recently, however, multiple technical breakthroughs have begun to allow large-scale analyses. These include advances in the sensitivity of mass spectrometry, stable isotopic labeling using amino acids in cell culture, developing variations on two-dimensional gels, labeling techniques to identify key proteins that are altered under different conditions, and the development of methods for isolating and sequencing small quantities of proteins (Elliott et al., 2004; Freeze, 2003; Ong et al., 2003). Proteomic analyses of NSCs have not yet been reported, despite the fact that cell lines have been available for several years.

IV. DATA MINING: CHROMOSOME MAPPING, PATHWAY ANALYSIS, AND DATA REPRESENTATION

A major problem with large-scale analysis has been knowing how to interpret the data, how to compare them, and how to extract meaningful and biologically relevant information. Biologists as a rule cannot simply look at long lists of genes to identify critical information, and examining the most abundant gene may not be of biological significance. For example, changes in notch or β-catenin signals of twofold or less may be biologically significant, whereas 10- or even 100-fold differences in the expression of some genes may be irrelevant for the biologic state of the cell. A particularly telling example of the relevancy of some differential gene expression data comes from the ESC literature, in which digital differential display identified TEX15 and DPPA5 as genes that are highly expressed in ESCs but low or absent in all other populations examined (Adjaye et al., 2005; Kim et al., 2005; Lagarkova et al., 2006). These results were verified by PCR and immunocytochemistry, although data from knockout mice showed that these genes are dispensable for all assessed functions (Amano et al., 2006). Alternatively, a similar strategy identified Nanog, a previously unknown key regulator of ESC differentiation (Chambers et al., 2003; Mitsui et al., 2003).

In the field, this has led to multiple attempts to consider how one should analyze data sets that are generated. In our laboratory, we have made the following assumptions: before pooling data or subjecting them to analysis, the quality of the sample used is tested. For example, with NSCs, the harvested sample is assessed for its expression of known NSC markers and the absence of markers of differentiation (to measure contamination; Figure 4.3, A). The presence or

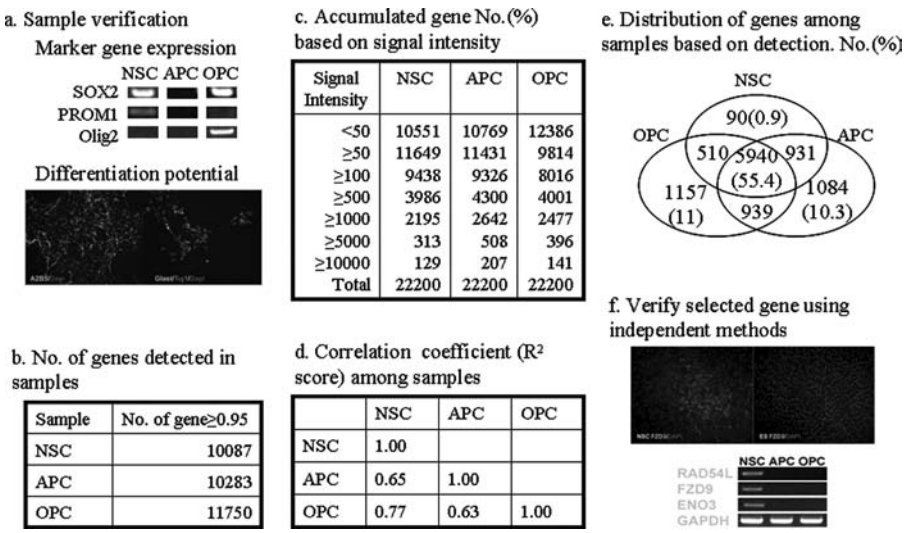

FIGURE 4.3 **A comparison of NSC, astrocyte precursor cells, and oligodendrocyte precursor cells using global gene expression analysis.** The process of profiling NSC is shown as an example. **A**, Astrocyte precursor cells and oligodendrocyte precursor cells are chosen as comparison groups, and the sample quality is monitored by examining marker expressions and their differentiation potential. **B**, Global comparison is shown in terms of gene numbers detected in each sample. **C**, The expressed gene numbers are subcategorized to show gene numbers in certain intensity. **D**, The relatedness among populations is accessed and shown by the R^2 score. **E**, The common and uncommon genes for each population are visualized in a Venn diagram. **F**, The results are verified by independent methods of polymerase chain reaction and immunocytochemistry. (See color insert.)

absence of these markers on the array is determined, and the ability of the array to detect such differences is assessed by running the same sample on an array. If an array hybridization or MPSS analysis fails to detect an expected result, then those data are not used, because all subsequent predictions are too uncertain. This analysis allows one to determine the expected sensitivity of the result, and it provides a rough idea of the sampling space (how much one will miss).

We then examine carefully the intensity distribution of the expression levels of the genes present on the array (Figure 4.3, B and C). We have noted that, in most cell types, the distribution is quite similar, and, therefore, any alteration suggests technical errors. Although normalization algorithms can be employed in an attempt to use a particular anomalous data set, we generally red flag it, because most normalization algorithms tend to skew results.

Empirically, we have determined that comparison across platforms is fraught with peril. Only positive results can be considered, and negative results are generally not interpretable. For example, ESC-derived NSC samples were taken and examined by MPSS and Illumina bead arrays, and a concordance of around 50% was shown, whereas the concordance rate for the sample run on a second Illumina array was close to 95% (unpublished observations). Even with such a comparison, only the presence or absence of an expression pattern can be considered, and no attempts to compare expression levels between different methods should be made. We have also determined that amplification tends to provide a different pattern than using unamplified RNA, even if the same sample is used. This appears to not be the result of operator error, because the amplification of different biologic replicates performed at different times yields more similar results than comparison between amplified and unamplified samples. Therefore, only samples that have been processed identically are compared as far as it is practical.

After we are comfortable with the quality of each sample processed, we examine differential gene expression by examining pairwise comparisons rather than pooling the data, or we compare them with a reference standard of baseline data that have been generated. This allows one to generate larger data sets and to compare across laboratories. For example, with NSCs, we suggest using human universal RNA as a potential reference standard: it is widely available, it is standardized, it has been run across multiple platforms, and such data sets are publicly available. This method allows one to readily determine if hybridization results are within the normal range and whether the data are usable and comparable with results from other laboratories.

After we have determined that we have a reasonable set of data, we then determine an appropriate cutoff for sensitivity with which we are comfortable. This ranges from array phenotype to phenotype, and it is an important criterion in any assessment. In MPSS, for example, a theoretical sensitivity when 3 million tags are sequenced is 3 transcripts per million (Miura et al., 2004a). However, we have empirically determined that testing or validating expression at such low levels is difficult, and, as such, it may or may not be useful to consider. In our hands, an expression level of 50 transcripts per million is readily verifiable, and it is a cutoff that we routinely use (Cai et al., 2006), although this does mean that we are potentially discarding useful information (which can be substantial, because a majority of genes are expressed at low levels). Likewise, with Illumina bead arrays, we use a cutoff of 50 to 100

arbitrary intensity units, although the theoretical sensitivity of the arrays is much lower. However, it is important that this be made clear in any publication or, alternatively, that the raw data be made available for independent analysis. This is particularly important because the genomic databases are constantly being curated, and expressed sequence tags assigned to a particular locus are being reassigned as better data become available. This curation sometimes means that a gene tag on an array may not represent the gene that it was originally thought to identify. Various groups have estimated this frequency, and they have recognized that it is an important source of error in these types of analyses. Therefore, we strongly recommend that such curation and updating be a regular part of the data analysis.

After we have determined a cutoff, established a set of phenotypes for analysis, and collected the curated data, we then can begin to consider how best to analyze the data. This analysis is dependent on the biologic questions one wishes to pose, and, although each strategy will be different, we can perhaps highlight some of the simple strategies that can be used. We currently feel that the genomic data that are most complete are those from the human and the mouse. Therefore, we have focused on large-scale analysis in these two species. We find that mapping the expressed genes onto a genome browser (chromosome mapping) provides a very useful overview of global patterns of gene expression. It allows one to study the regulation of genes on a global scale, to identify genomic hotspots, and to correlate with known chromosomal breakpoints, single-nucleotide polymorphism data sets, data developed by groups working in different disciplines. We would recommend the University of California, San Francisco golden path browser for this purpose.

A second important strategy that we routinely use is what we call a "pathway analysis." Here, rather than looking at the expression of an individual gene, we examine an entire signaling pathway by mapping the expression of all detected genes for that pathway. A visual representation of the "on" and "off" state of the pathway can be easily obtained, and observation of the pathway as cells differentiate can provide a much clearer look at whether genes in that pathway are important in the process of differentiation. For example, such a pathway analysis clearly identified the LIF/GP130 pathway as being important in the NSCs of humans but not of mice (unpublished data). It also helps identify key genes that must be tested in verification studies. Multiple commercial programs to perform such pathway analyses exist, and we recommend using any one of these.

Overall, our experience has been that, if an effort is made to develop well-curated data sets, the verification of observations is generally greater than 50%, and one can glean important and reliable information. Although a hit rate of 50% may sound low, it is useful to consider that this is much better than the 1 in 30,000 chance of finding a functionally useful gene that one started off with before analysis. For example, there are currently about 10 NSC biomarkers that have been identified to date. However, using a large-scale analysis, one could readily identify perhaps 200 such markers, of which perhaps 25% (50) would be novel or unexpected genes. With a hit rate of 50%, one could identify 25 new markers during a 3-month experiment, thus more than doubling the number of known NSC markers. In the next section, we will discuss some general observations made about NSCs.

V. GENERAL OBSERVATIONS ABOUT THE PROPERTIES OF NEURAL STEM CELLS

We find that NSCs appear to be similar to other cells in that they synthesize about 10,000 to 12,000 genes of an estimated 35,000 or 40,000 genes annotated in the RefSeq database. Average total RNA per cell tends to be higher among stem cells as compared with other cells (average, 5–10 pg), but it is similar to levels seen in metabolically active cells. The distribution of transcript frequency suggests that most genes are transcribed at relatively low levels (<50 transcripts per cell) as assessed by MPSS. Mitochondrial, ribosomal, and housekeeping genes tend to be more abundant, whereas transcription factors, growth factors, and other cell-type–specific molecules are expressed at much lower levels. This pattern of gene expression is similar to that seen among most other cell types. The comparison of data from other cell types suggests that, on average, the majority of genes are shared or held in common, whereas approximately 20% are different (by greater than 10-fold) in any two samples. The mapping of all expressed genes does not show a chromosomal bias, which has been suggested in other stem cell populations. An example from an array experiment performed in our laboratory comparing NSCs, astrocyte precursor cells, and oligodendrocyte precursor cells is shown in Figure 4.3. The average correlation rate among NSC cell populations grown under different conditions was 0.90 (0.79–0.99), whereas the score dropped to 0.65 (0.57–0.71) and 0.77 (0.76–0.80) when NSCs were compared with astrocyte precursor cells and oligodendrocyte precursor cells, respectively. The correlation is much closer when identical samples are compared in two independent sequencing runs to 0.99 (0.96–0.99). By contrast, when identical samples are compared between two methods (serial analysis of gene expression and MPSS or expressed sequence tag and MPSS), then the concordance rates are much lower (0.7), which suggests that those genes that are common between two methods are likely to be important. However, the lack of a high concordance when identical samples are analyzed by different methodologies suggests that caution should be taken when assuming that the failure to detect expression by any one method means that a result is valid.

Examining NSC-enriched genes suggests that several major pathways are active in NSCs (Abramova et al., 2005; Cai et al., 2006). These include the LIF/gp130 (in humans only), the fibroblast growth factor signaling pathway, the cell cycle regulatory pathways (including myc and DNA repair), and anti-apoptotic pathways. Intriguingly, genes regulating the timing of differentiation, antisense RNA, small interfering RNA, specific methylases, and chromatin remodeling enzymes appear to be present at high levels, which suggests that epigenetic remodeling is an important aspect of NSC biology, as has been shown for ESCs as well.

A. Species Differences

An important finding that has been made clear from the availability of data sets from both mouse and human ESCs is the variability between species. Although many key pathways are conserved, many differences have been highlighted as well. For example: 1) Oct3/4 homologues likely do not exist in chicken embryos (Soodeen-Karamath et al., 2001); 2) LIF signaling, which is critical for ES cell self-renewal in rodents, does not appear to be critical or even required for human ESCs (Daheron et al., 2004; Ginis et al., 2004;

Niwa, 2001); and 3) no paralogs of E-Hox have been identified in humans, and ESC-expressed Ras appears to be a pseudogene in humans (Bhattacharya et al., 2004). The low overall concordance rate between human and rodent ESCs (in one comparison, around 40%) relative to that seen in human-to-human cell comparisons (90% between human ESC samples) provides additional support for these findings (Wei et al., personal communication).

B. Lack of A Stemness Phenotype

There is an operating bias in the literature that most stem cells will be similar to each other, and, indeed, several manuscripts simply use the term "cell" rather than specifying which tissue they arise from and the stage of development used. This bias, however, is not supported by the large-scale analytical data. Comparisons of NSCs with other populations of stem cells have not identified any common stemness pathway (Cai et al., 2004a). Another important finding that has become obvious from such data set examination is the lack of any common stemness genes between ESCs and other somatic stem cells. Comparing data sets with expression in NSCs does not identify a common subset of genes, and it suggests that NSCs are quite different from other somatic stem cells (Fortunel et al., 2003; Ivanova et al., 2002; Ramalho-Santos et al., 2002). Our recent results comparing ESC-derived NSCs with fetal-derived NSCs (Shin et al., 2007) and more limited comparisons (Pevny and Rao, 2003) have shown clearly that stem cells can be readily distinguished from each other and that stem cells or progenitor cells harvested at different stages of development behave differently.

C. Allelic Variability

It is clear that, although individual stem cells share properties such as the ability to self-renew in culture and to differentiate into various phenotypes, there will nevertheless be differences between individual cell lines based on the genetic profile of the individual from whom they were derived. These allelic differences are not unexpected, and they merely reflect the diversity of phenotypes seen in the human population. Perhaps what has not been appreciated as much is how much variability this might impose on stem cell behavior, even if care is taken to isolate cells from the same region at the same developmental time using methods that are as similar as possible. Consequences of such allelic variability have been described in allergic responses and the ability to smell different odors, metabolize alcohol, digest milk, or respond to toxins (Sultatos et al., 2004; Usuku et al., 1992).

When global pairwise comparisons are made among multiple stem cell lines using the same platform, one sees a high reproducibility when the same sample is run repeatedly (correlation coefficients in the range of 0.98–0.99). However, when two samples isolated by the same group at around the same time are run, the differences are much larger (correlation coefficient, 0.92–0.94), which indicates that a significant number of genes are differentially expressed between two stem cell lines. This sort of difference is consistently seen in all stem cell populations, it is generally less than the difference between a cell line and its differentiated progeny, and it is less than the difference between a cell line from one species as compared with another (Ginis et al., 2004). Nevertheless, at the individual gene level, such changes can be

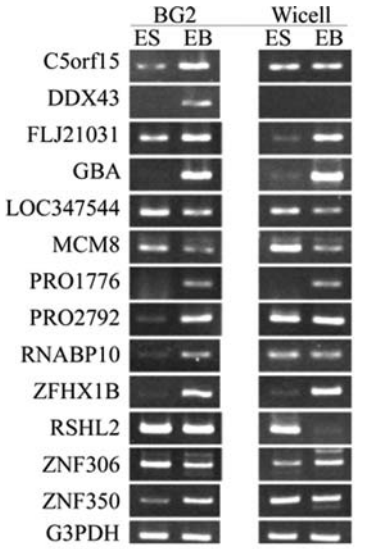

FIGURE 4.4 Allelic differences are shown in two different ESC lines (modified from Bhattacharya [2005]). Total RNA is isolated from embryonic stem cells and embryoid bodies of two different ESC lines (BG02 and Wicell), and the differences are confirmed by reverse transcriptase polymerase chain reaction. Note the large differences in gene expression.

quite dramatic; this is shown in Figure 4.4, which compares ESC lines (Bhattacharya et al., 2005). Such allelic variability likely underlies the differences in propagation, self-renewal, and the ability to differentiate into specific phenotypes that have been reported.

Large-scale analysis of the kind we have reported allows one to map the underlying basis of such variability and to make predictions about the behavior of cell lines (Lo et al., 2003; Ross et al., 2000; Yan et al., 2002a; Yan et al., 2002b). For example, Lo et al. (2003) demonstrated a reliable measurement of allele-specific gene expression based on large-scale analysis. Selected genes were not only in the imprinted regions, but they were also distributed throughout the genome, which may be responsible for phenotypic variation.

Thus, as one begins considering stem cells for therapy, one will need to carefully assess whether a lot of NSCs prepared from one individual will behave similarly as another lot of cells prepared from a different donor, even if identical protocols were used and the experiments were performed in a similar fashion. This inherent variability is of major concern when evaluating cells as therapy.

D. Age-Dependent Characteristic Changes of NSCs

The examination of human cells in culture has shed some light onto the cellular basis of aging. When grown in culture, normal human cells will undergo a limited number of divisions before entering a state of replicative senescence, during which they remain viable but are unable to divide further (Miura et al., 2004b). This change has been attributed to changes in mitochondria, protein expression, the loss of genomic integrity, oxidative damage, and the

progressive loss of DNA repair ability or the erosion of telomere ends. Results from our laboratory and others have shown that ESCs appear to be immortal and that they can be propagated in continuous culture for a period of at least 2 years. The examination of the expression of immortality-associated genes has suggested that the expression of key regulators is important (Miura et al., 2004b). These include the expression of telomere-associated proteins, the expression of some immortality-associated genes, high levels of DNA repair enzymes, the inhibition of p53, and the absence of retinoblastoma protein, thus altering cell-cycle regulation. Comparing the phenotype of such genes between NSCs and ESCs shows significant differences. NSCs have shown the active expression of Rb, and their pattern of expression of telomere-related genes and immortality-associated genes differs from that of ESCs (Table 4.4). The difference is also apparent between adult stem cell populations and ESCs, which suggests that NSCs are not easily propagated indefinitely in culture. Indeed, Whittemore and colleagues have shown that the long-term propagation of NSCs in culture decreased their ability to differentiate (Quinn et al., 1999). Pruit and colleagues have shown that NSCs undergo karyotypic changes as the animal ages, thus providing further confirmation of this hypothesis (Bailey et al., 2004). Our recent results examining the karyotypic stability of NSCs in culture have indicated that NSCs are less stable in culture than ESCs and that they can acquire karyotypic changes in as few as

TABLE 4.4 Immortality-Associated Genes and Their Expression in Embryonic Stem Cells and Neural Stem Cells

Gene Symbol	Description	Embryonic Stem Cells	Neural Stem Cells
TP53	Tumor protein p53	118	65
TERF1	Telomeric repeating binding factor 1	2163	357
TERF2	Telomeric repeating binding factor 2	508	1993
TINF2	TERF1 interacting nuclear factor 1	488	937
TERF2IP	TERF2 interacting protein	876	4252
RAD50	RAD50 homolog	38	2
Rif1	Telomere-associated protein RIF1 homologue	61	201
MRE11A	MRE11 homologous A	46	169
POT1	Protection of telomeres 1	78	199
TEBP	Unactive progesterone receptor, 23 kD	3561	5473
TEP1	Telomerase-associated protein 1	377	1983
TERT	Telomerase reverse transcriptase	23	3
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21)	1434	6303
CDKN2A	Cyclin-dependent kinase inhibitor 2A (p16)	91	188
CDK4	Cyclin-dependent kinase 4	771	2177
CCND1	Cyclin D1	1438	2655
CCND2	Cyclin D2	3063	6363
CCND3	Cyclin D3	183	342
RB1	Retinoblastoma 1	33	188
E2F1	E2F transcription factor 1	36	136
PIK3CG	Phosphoinositide 3 kinase, catalytic, gamma polypeptide	0	36

The gene expression of embryonic stem cells and embryonic-stem-cell–derived neural stem cells was shown in an arbitrary unit (Shin et al., 2007).

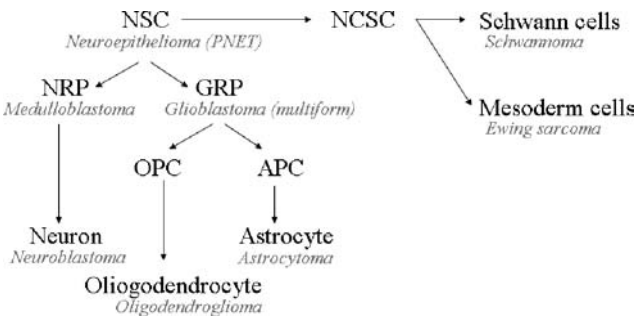

FIGURE 4.5 Hierarchical cell lineage relationships and the possible counterparts of a transformed tumor population. *NSC,* Neural stem cells; *PNET,* primitive neurectodermal tumour;

10 passages. Overall, large-scale analysis suggests that there are fundamental differences in cell-cycle and immortality-associated genes and that these differences predict that NSCs age in culture and that they will senesce, as do other adult stem cell populations.

VI. CANCER STEM CELLS

An exciting finding has been the discovery that many cancers may be propagated by a small number of stem cells present in the tumor mass (Al-Hajj et al., 2003; Huntly et al., 2005; Reya et al., 2001; Singh et al., 2004). This was first described in breast cancers and, subsequently, in a variety of solid tumors. In the nervous system, several reports have suggested that cancer stem cells can be identified and that these cells bear a remarkable similarity to the NSCs present in early development (Hemmati et al., 2003; Singh et al., 2004). Likewise, cells resembling glial progenitors have been isolated from some glial tumors (Kondo et al., 2004; Noble et al., 1995). We and others have suggested that tumors perhaps can be phenotypically classified, and this classification correlates well with a cell of origin identified on the basis of findings from lineage relationships that exist between stem and progenitor cells (Figure 4.5). The advantage of having a detailed database of gene expression from multiple normal cell lines and progenitor cells and the ability to compare their gene expression profiles with their transformed counterparts is obvious. One could identify core conserved pathways and key differences and use transforming regimes with normal stem cell populations to develop tools to probe dysregulated pathways. These experiments are not technically difficult, and they would depend on the development of an adequate database for mining. Efforts along this path have already been initiated, and several groups have begun to report about the properties of undifferentiated NSCs and to isolate cancer stem cells from appropriate tumors. We anticipate that important insights will be gained by such an analysis.

VII. APPLICATIONS OF PROFILING DATA

Overall, the initial efforts at profiling NSCs from rodents and humans have yielded useful insights, and they have allowed for the identification of key regulatory pathways and novel genes that are likely to play a role in

regulating ESC self-renewal. The data sets developed to date are an important resource, and they can be mined with readily available tools. Efforts are underway to provide all of this data in a readily accessible format that would allow one to be able to examine the pattern of expression of his or her favorite gene.

Although profiling information has already yielded fruit in terms of understanding the basic biology of the cells, we believe that the databases that are being developed will have a utility far beyond current applications. The implications of some general findings are discussed below.

1. *Generating stable cell lines will be difficult, and screening will require doing so in multiple cell lines.* Two important observations from the current body of data are relevant. First, the stem cell state is not akin to the state of transformed cells (i.e., some stem cells may be immortal, but they use mechanisms that are not identical to those coped by transformed cells). The logical extension of this observation is that immortalization and developing cell lines will not be a trivial task. Indeed, despite the fact that stem cells were isolated from the nervous system more than 15 years ago, few cell lines are available. The most commonly used line, C17.2, is karyotypically abnormal, and it does not truly reflect the properties of nonimmortalized cells. The second clear conclusion from current work is that, although various types of NSCs are similar overall, differences exist, and, in many cases, the differences are quite large. Thus, any screening application will need to take into account such allelic variability, and it will require the screening of multiple lines.
2. *Results from fetal tissue and ESCs will not necessarily predict the behavior of adult stem cells.* The comparison between ESC-derived NSCs and the more limited comparisons among neuroepithelial NSCs, neurosphere-forming cells, and stem cells harvested from the adult all show that cells differ depending on the stage at which they are isolated, the time and number of passages in culture, and the environmental influences on aging (which in turn are dependent to some extent on the genomic phenotype of the cell). These differences predict that extrapolation from one population to another will be difficult, and extreme caution will need to be exercised while making conclusions from the available data.
3. *Mouse models may not accurately predict behavior in humans.* The species differences highlighted between mice and rats, between mice and humans, and among different primate lines suggest that transplanting human cells into a xenograft model is unlikely to accurately reflect the response of cells in a human. Furthermore, because intrinsic differences exist among species, human-into-human experiments cannot be modeled by performing rat-into-rat or mouse-into-mouse experiments, either. Although much can be learned from such experiments, it is also clear that no model will be absolutely accurate, so care must be taken when one derives conclusions on the basis of the results from different species.
4. *Transdifferentiation will be difficult.* A final important conclusion based on cross–stem-cell comparisons is that adult stem cells harvested from various regions are no more similar to each other than they are to differentiated progenies from them. Furthermore, adult stem cells differ significantly from ESCs in many of their properties. This suggests that transdifferentiation will be difficult.

The implications of these observations for drug discovery and therapeutic applications of stem cells are obvious, because they suggest that, although substantial progress has been made, much remains to be done. In addition, if one does not carefully design one's experiments to take into account species and allelic variability, then one may arrive at conclusions that are not warranted.

SUMMARY

- Large-scale genomic analysis has provided unique insight into the biology of NSCs. The wealth of genomic data and the multiplicity of available cell lines have enabled researchers to identify critical conserved pathways that regulate self-renewal and to identify markers that tightly correlate with the NSC state.
- Comparison across species has suggested additional pathways that are likely to be important in the long-term self-renewal of NSCs, and the meta-analysis of existing data sets has provided additional unique insights.
- Newer technologies have provided sophisticated tools to probe into aspects of cell biology that were difficult to study previously.
- Combining these technologies and pooling information generated provides a synergistic increase in the value of the available information, and it provides a platform for future breakthroughs.

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Useful Web Sites (Suppliers Providing Array-Based Products and Services)

Supplier	Products and services	Web site
Illumina	SNP analysis, gene expression profiling	http://www.illumina.com
Invitrogen	microRNA, genomic profiling	http://www.invitrogen.com
Superarray	Focused array	http://www.superarray.com
Solexa	MPSS	http://www.lynxgen.com/
Affymetrix	Microarray	http://www.affymetrix.com/

5
EPIGENETIC INFLUENCES ON GENE EXPRESSION PATHWAYS

SUNDEEP KALANTRY and TERRY MAGNUSON

Department of Genetics and the Carolina Center for the Genome Sciences, University of North Carolina, Chapel Hill, NC

INTRODUCTION

During cell division, the parental cell transfers genetic as well as epigenetic information to daughter cells. In broad terms, epigenetic inheritance encompasses all transmitted cellular material other than DNA. Examples include proteins and RNAs present in oocytes that persist beyond fertilization through early embryonic development, and transcription factors that induce additional copies of their own in descendant cells. More commonly, however, the term *epigenetics* refers to an increasing number of reversible covalent modifications of DNA and proteins that influence various DNA-based processes, such as DNA repair, recombination, replication, and transcription. Of these, epigenetics is arguably most often defined as the changes in gene expression that are stably transmitted to descendant cells without alterations in the DNA sequence. By establishing and propagating patterns of gene expression through cell division, epigenetic regulation has the ability to contribute to the maintenance of a cell's identity, a phenomenon referred to as *cellular memory* (Cavalli, 2006).

I. EPIGENETICS AND CELLULAR MEMORY

Evidence increasingly indicates that cells employ epigenetic transcriptional regulation at all developmental levels, including in plasticity, proliferation, cell fate decisions, differentiation, and disease. Prominent examples include the propagation of embryonic stem cells (ESCs); epigenetic mechanisms allow ESC self-renewal rather than the production of a differentiated population (Cavalli, 2006). Additionally, epigenetic regulation may also act to distinguish differentiated cell types from each other (Lin and Dent,

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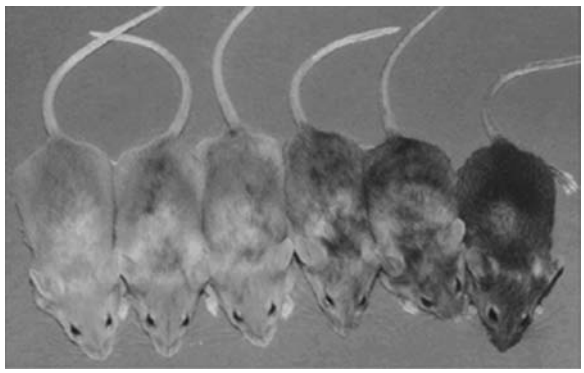

FIGURE 5.1 **Genetically identical A^{vy}/a mice with a range of coat colors from completely yellow to a mixture of yellow/agouti to completely agouti.** (From Morgan et al., 1999.)

2006). Inherent in a cell's ability to modulate its developmental potential is a capacity to change its epigenetic profile.

A striking example of epigenetic influence is provided by the coat color markings of inbred mice carrying the agouti yellow viable (A^{vy}) allele. Although genetically indistinguishable, these mice differ in the color of their fur. Although some mice are fully yellow, others are variegated yellow with some spots of agouti, and still others are fully agouti (Figure 5.1). Moreover, this variability correlates with the coat color of the mother: a female with a yellow coat will generate a higher percentage of yellow offspring (Blewitt et al., 2006). Alternatively, the coat of the father has no effect on the color of the pups. It is hypothesized that a variable epigenetic effect on the A^{vy} allele in the maternal germ line results in differences in the coat color phenotypes in the ensuing generation (Blewitt et al., 2006).

II. EPIGENETIC REPROGRAMMING DURING DEVELOPMENT

Similarly, the coat color of the first cloned cat, called Cc (for either "copy cat" or "carbon copy"), is different from that of her genetically identical donor (Shin et al., 2002). In this case, the differences in coat color markings can be attributed to X-chromosome inactivation (XCI). XCI is a paradigmatic epigenetic phenomenon that results in the transcriptional silencing of genes on one of the two X chromosomes in female mammals, resulting in equivalence of X-linked gene dosage between males and females (Heard, 2005). XCI is enacted stochastically in individual embryonic cells, and it is clonally transmitted such that all descendant cells maintain silencing of the same X chromosome. The genetic mosaicism conferred by XCI is the cause of the unexpected coat color of Cc. The two Xs in Cc carry different alleles of a pigmentation gene that contributes to the calico coat color. The stochastic nature of XCI results in a dissimilar pattern of pigmentation in Cc and her donor. Therefore, the two animals have a unique distribution of cells harboring a different active allele of the pigmentation gene.

Although remarkable, the cloning of animals by somatic cell nuclear transfer is a very inefficient process, with only 1% to 3% of all transferred nuclei resulting in healthy adult animals (Armstrong et al., 2006). The low rate of success is thought to be partly the result of aberrant genomic reprogramming

of donor nuclei. The proper spatial and temporal expression of genes during the development of the cloned embryo requires a resetting of the epigenetic profile of the donor nucleus. The epigenetic modifications that characterize the somatic nucleus must be reconfigured such that appropriate sets of genes are expressed in the developing cloned embryo. These changes must occur in a considerably smaller window of time in a cloning experiment (at most several hours) as compared with the reprogramming that normally occurs during the formation of mature germ cells in animals (weeks to months). Evidence from mouse cloning experiments supports this idea; nuclei from more differentiated mouse cells are less efficient donors than cells from early embryos. Clones that survive to term from transferred nuclei from blastomeres of two-, four-, and eight-cell stage embryos decreased from 22% to 14% to 8%, respectively (Cheong et al., 1993; Wilmut et al., 2002). It may not be a coincidence that the first somatic cell nuclear transfer cloning success of a differentiated nucleus occurred in sheep. In cattle, gene expression in zygotes begins later than in mice (morula vs. two-cell stage; Thompson, 1996). This later activation of the cattle genome allows more time for efficient reprogramming of the transplanted chromatin by signals from the oocyte cytoplasm. The importance of epigenetic reprogramming during cloning is underscored by the finding that even adult mice derived by nuclear transfer harbor epigenetic aberrations (Humpherys et al., 2001). In these clones, the expression of imprinted genes (a unique set of genes that are preferentially expressed through cell division from either the maternal or the paternal allele but not both) is found to be dysregulated.

III. EPIGENETIC CONTROL OF IMPRINTED GENE EXPRESSION

The parent of origin-specific bias in the expression of imprinted genes offers another notable example of epigenetic regulation. The existence of imprinted genes violates classical Mendelian genetic theory, which postulates the equal inheritance of and predictable segregation of genetic characteristics among the progeny. Animal breeders have long known, however, that the reciprocal mating of some animals yields phenotypically different progeny. One well-known example is the interspecific cross between a donkey and a horse, which produces either a hinny (when the donkey is the mother) or the mule (when the horse is the mother). These apparent exceptions to Mendel's laws led to the speculation that there might be parent-of-origin differences in the gametes within the same species (Monk, 1995). The inequality of maternal and paternal genomes was cemented by the experimental generation of mouse embryos that contained a diploid complement of only maternal chromosomes (gynogenones or parthenogenones) or paternal chromosomes (androgenones; McGrath and Solter, 1984; Surani and Barton, 1983). Both sets of embryos died during gestation, albeit with disparate defects. Whereas gynogenones and parthenogenones displayed the preferential development of embryonic cell types, androgenones gave rise disproportionately to extra-embryonic cells of the trophectoderm lineage. Similar parental genome differences were also indicated through the phenotypes of mice carrying partial uniparental disomies in the form of Robertsonian translocations (Cattanach and Kirk, 1985). Animals that received both homologs of certain chromosomes or subchromosomal regions from one parent (and none from the other)

displayed developmental defects, such as increased or retarded growth and embryonic lethality, which were not manifested in reciprocal crosses. In both sets of experiments, the divergent defects in the two classes of embryos highlighted the epigenetic differences in the parental genomes.

Imprinting's raison d'être has been proposed to lie in a tug of war between the sexes. Put forth by Moore and Haig, this theory postulates that whereas a male wants to ensure that its offspring develop preferentially in a female that may simultaneously bear offspring sired by other males, a mother would prefer to maximize the chances of transmitting its genome by having multiple offspring (Moore and Haig, 1991). One prediction of this theory, therefore, would be that paternally expressed imprinted genes would extract maternal resources during gestation, thus resulting in an enlarged embryo; maternally expressed genes, on the other hand, would limit such growth. The phenotypes of mice lacking the reciprocally-imprinted genes *Igf* (Insulin growth factor) and *Igf2r* (Insulin growth factor receptor 2) lend support to this battle-of-the-sexes theory. *Igf* is paternally expressed, and the transmission of its null allele from the sire results in an underdeveloped embryo (maternal transmission does not yield a phenotype, because the maternal allele is silent; DeChiara et al., 1991). *Igf2r* is preferentially expressed from the maternal allele, and a null allele inherited from the mother causes an enlarged fetus (Barlow et al., 1991).

Imprinted genes are not always monoallelically expressed; they may be subject to tissue-specific or developmental-stage–specific regulation (see the Recommended Resources section). The transcriptional bias among alleles of imprinted genes, however, must be reset in the germline of each sex such that either allele has the potential to be recognized as maternal or paternal upon transmission to the subsequent generation. By extension, then, allelic preference in the expression of imprinted genes must be a readout of epigenetic marks established in the parental germline.

IV. MECHANISMS OF EPIGENETIC REGULATION

Eukaryotes employ two common modes of epigenetic gene regulation, both of which involve reversible covalent modifications of chromatin components. *Chromatin* is defined as a complex of DNA and proteins, primarily histones, that the cell uses to pack a large amount of DNA into the small volume of the nucleus. The fundamental unit of chromatin is the nucleosome, which is comprised of 147 base pairs of DNA wrapped around a core of histones H2A, H2B, H3, and H4. Nucleosomes are bundled into 30-nanometer fibers that are in turn compacted into higher-order structures of chromatin that form the chromosome. To achieve transcriptional activation or the stable silencing of genes, the organism must employ strategies to surmount this complex packaging.

Gene expression in eukaryotes is regulated by a coordinated interplay of chromatin-remodeling proteins, general and gene-specific transcription factors, RNA polymerases, and DNA and histone modifying factors. Although chromatin-remodeling enzymes in combination with general and gene-specific transcription factors can induce gene expression during interphase, the propagation of transcriptionally silenced states that survive mitosis or meiosis is performed by DNA and histone-modifying proteins

(histone modifications, along with the incorporation of variant histones, may also be a mechanism by which transcriptionally active regions of the genome are marked to persist through cell division). The modifications of chromatin brought about by these factors result in either the recruitment of specific protein complexes or alterations in nucleosome structure, which in turn directly or indirectly influence the activity of the basal transcription machinery (Craig, 2005).

A. DNA Methylation

The prototypic epigenetic modification is the methylation of DNA cytosine residues. Cytosine methylation is most often found in cytosine–guanine dinucleotides (CpG), and it is present in many eukaryotes, including in all mammals (Goll and Bestor, 2005). CpGs are overwhelmingly found in transposons, which are interspersed repetitive sequences that constitute more than 38% of the mouse genome (and 46% of the human genome). The methylation of CpGs in transposons is proposed as a mechanism to suppress transposon reactivation and thereby prevent genomic instability. CpGs are underrepresented elsewhere in the mammalian genome, possibly as a result of the mutation of methylated cytosine by deamination to thymidine, with the notable exception of 5' ends of the majority of genes. The methylation of CpGs in these regions—often promoters—has long been thought to repress transcription. Most of the evidence supporting this notion has occurred through experiments in established cultured cells. Loss-of-function experiments in mice, however, indicate that CpG methylation in the promoter regions of genes does not act as a general inhibitor of gene expression *in vivo* (described below). Consistently, most CpGs in both transcriptionally active and silent genes are unmethylated (Walsh and Bestor, 1999). Although DNA methylation appears to be dispensable for general transcriptional inhibition, it has been shown to be necessary for the proper regulation of imprinted gene expression and for the maintenance of silencing of the inactive X chromosome.

Cytosine methylation is mediated by an evolutionarily conserved set of proteins called *DNA methyltransferases* (Dnmts), which catalyze the transfer of methyl groups onto the fifth position of cytosines. Dnmts have been classified into two groups: *de novo* and maintenance methyltransferases. Whereas maintenance DNA methylases propagate preexisting methylation patterns by methylating the complimentary strand of hemimethylated DNA after DNA replication, *de novo* methylases establish those patterns.

Five Dnmts have been identified in mice, all of which have been subject of much *in vitro* as well as *in vivo* scrutiny. The first eukaryotic Dnmt to be purified and cloned, *Dnmt1,* was found to have a preference for methylating hemimethylated DNA (Goll and Bestor, 2005). This activity led to *Dnmt1* being designated as a maintenance DNA methylase, although it does methylate unmethylated DNA substrate to some extent. Several mutant alleles of *Dnmt1* have been generated in mice. *Dnmt1* homozygous-null ESCs and embryos are severely demethylated, although methylation persists at a low level (Lei et al., 1996). The mutant embryos display perturbations in imprinted gene expression and XCI (Table 5.1). Although some imprinted genes, such as *H19* and *Kcnq1ot1,* are expressed from both alleles, others, such as *Igf2* and *Kcnq1,* are expressed from neither (Li et al., 1993). The inactive X chromosome in mutant embryos is reactivated in some somatic cells, which results in two active X chromosomes

in females (Sado et al., 2000). *Dnmt1*$^{-/-}$ embryos also express increased levels of long terminal repeat retrotransposon expression (Walsh and Bestor, 1999).

Despite the massive reduction in DNA methylation, *Dnmt1*$^{-/-}$ embryos develop normally until embryonic day 8.5. Moreover, gene expression analysis in cultured *Dnmt1*$^{-/-}$fibroblast cells, which are derived from *Dnmt1* conditional-null embryos, identified a much smaller fraction of dysregulated genes as compared with the percentage that harbor CpG promoter methylation (Jackson-Grusby et al., 2001). The late lethality of *Dnmt1*$^{-/-}$ embryos and the limited defects in gene expression, therefore, argue against a general transcriptional-inhibitory function for DNA methylation. On the other hand, the low level of methylation observed in the *Dnmt1*$^{-/-}$ embryos may be sufficient for the repression of most genes subject to methylation, and it perhaps explains the late lethality of the mutant embryos.

Dnmt2, which is the most highly conserved and widely found Dnmt, lacks detectable methylase activity in both genetic and biochemical tests. Despite containing consensus methyltransferase motifs, it has failed to display the methylation of either hemimethylated or unmethylated DNA (Goll and Bestor, 2005). Additionally, mice lacking *Dnmt2* are phenotypically normal (Table 5.1; Goll et al., 2006).

TABLE 5.1 Phenotypes of Mice with Mutations in Chromatin-Modifying Proteins

	DNA Methyltransferases
Dnmt1$^{-/-}$	Developmental arrest at embryonic day 8.5; severe demethylation of CpG cytosines; increased retrotransposon expression; dysregulated expression of some imprinted genes; reactivation of the inactive X chromosome in some somatic cells (Li et al., 1993; Li et al., 1992; Sado et al., 2000)
Dnmt2$^{-/-}$	No obvious phenotypic defects (Goll et al., 2006)
Dnmt3a$^{-/-}$	Mice born runted with lethality at 4 weeks of age; males have diminished germ cells; conditional mutation reveals requirement in the establishment of male-specific methylation marks in some imprinted genes (Hata et al., 2002; Okano et al., 1999)
Dnmt3b$^{-/-}$	Mid- to late-gestation lethality (embryonic days 14.5 to 18.5); neural tube defects; demethylation of minor satellite repeats (Okano et al., 1999)
Dnmt3a$^{-/-}$, *Dnmt3b*$^{-/-}$	Developmental arrest at embryonic day 8.5, with defects in both de novo and maintenance methylation (Okano et al., 1999)
Dnmt3l$^{-/-}$	Mice born but sterile; required in both male and female germ cells to establish proper methylation patterns (Bourc'his and Bestor, 2004; Bourc'his et al., 2001; Hata et al., 2002)
	Methyl–CpG Binding Proteins
Mbd1$^{-/-}$	Viable, with subtle neurologic defects (Zhao et al., 2003)
Mbd2$^{-/-}$	Viable, with defects in maternal behavior (Hendrich et al., 2001)
Mbd3$^{-/-}$	Postimplantation stage developmental arrest, with lethality by embryonic day 8.5 (Hendrich et al., 2001)
Mecp2$^{-/-}$	Postnatal lethality at 6 to 12 weeks of age, with an array of neurologic defects (Chen et al., 2001; Guy et al., 2001)
	Histone Acetyltransferases
Gcn5(Pcaf-b)$^{-/-}$	Midgestation lethality (embryonic days 9.5 to 11.5), with mesoderm defects (Xu et al., 2000; Yamauchi et al., 2000)
Pcaf$^{-/-}$	Viable, with no apparent defects (Xu et al., 2000; Yamauchi et al., 2000)
p300$^{-/-}$	Midgestation lethality (embryonic days 9.5 to 11.5), with exencephaly and cardiac defects (Yao et al., 1998)

(Continued)

TABLE 5.1 Phenotypes of Mice with Mutations in Chromatin-Modifying Proteins—Continued

Cbp$^{-/-}$	Midgestation lethality (embryonic days 9.5 to 10.5), with neural tube, hematopoietic, and vascular defects (Oike et al., 1999)
p300$^{-/-}$, *Cbp*$^{-/-}$	Midgestation lethality (Yao et al., 1998)
	Histone Deacetylases
Hdac1$^{-/-}$	Midgestation lethality (embryonic days 9.5 to E10.5), with cell proliferation and growth defects (Lagger et al., 2002)
Hdac9$^{-/-}$	Viable, with cardiac hypertrophy apparent at 8 months of age (Zhang et al., 2002)
Sir2a$^{-/-}$	Neonatal lethality in inbred and sterility in outbred mice (McBurney et al., 2003)
	Histone Modifications
G9a$^{-/-}$	Developmental arrest at embryonic day 8.5, with loss of euchromatic H3–K9 methylation (Tachibana et al., 2002)
Eu-HMTase (GLA)$^{-/-}$	Developmental arrest and lethality at around embryonic day 9.5, with loss of H3–K9 monomethylation and dimethylation (Tachibana et al., 2005)
Eset$^{-/-}$	Peri-implantation stage lethality (embryonic days 3.5 to 5.5), with defects in embryonic cell proliferation (Dodge et al., 2004)
Suv39h1$^{-/-}$, *Suv29h2*$^{-/-}$	Low-penetrance lethality of embryos at around embryonic day 14.5; male sterility; loss of H3–K9 methylation of pericentric heterochromatin; genomic instability (Peters et al., 2001)
	Polycomb Group Proteins
Eed$^{-/-}$	Midgestation lethality (embryonic days 8.5 to 10.5), with defects in imprinted X-chromosome inactivation and autosomal imprinted gene expression (Faust et al., 1995; Kalantry and Magnuson, 2006; Kalantry et al., 2006; Mager et al., 2003; Shumacher et al., 1996; Wang et al., 2001)
Eed$^{hypo/hypo}$	Homeotic transformations (Shumacher et al., 1996; Wang et al., 2002)
Ezh2(Enx1)$^{-/-}$	Postimplantation embryonic lethality at embryonic day 5.5 or 8.5 (O'Carroll et al., 2001)
Yy1$^{-/-}$	Peri-implantation lethality, with defects in embryonic cell proliferation (Donohoe et al., 1999)
Bmi1$^{-/-}$	Postnatal lethality, with homeotic transformations, hematopoietic defects, seizures, and ataxia (Jacobs et al., 1999; van der Lugt et al., 1994)
M33$^{-/-}$	Homeotic transformations and sex reversal of males to females (Core et al., 1997; Katoh-Fukui et al., 1998)
Mel18(Zfp144)$^{-/-}$	Postnatal lethality at 4 weeks of age and homeotic transformations (Akasaka et al., 1996)
Ring1a$^{-/-}$	Homeotic transformations (del Mar Lorente et al., 2000)
Mph1(Rae28)$^{-/-}$	Neonatal lethality, with homeotic transformations and neural crest defects (Takihara et al., 1997)
Ring1b(Rnf2)$^{-/-}$	Postimplantation lethality at embryonic day 7.5, with defects in cardiac mesoderm production (Voncken et al., 2003)

CpG, Cytosine–guanine dinucleotides; *H3-K9,* histone H3–lysine 9.

Mammals harbor three methyltransferases of the *Dnmt3* family: *Dnmt3A, Dnmt3B,* and *Dnmt3L*. Whereas *Dnmt3A* and *Dnmt3B* can methylate both hemimethylated and unmethylated DNA at equal rates, *Dnmt3L* lacks functional methyltransferase activity. Mice deficient in *Dnmt3A* survive to term, but they are born runted and die a few weeks later, and they show a loss of germ cells in males (Table 5.1; Okano et al., 1999). Preferential deletion by conditional mutagenesis in germ cells demonstrated that *Dnmt3A* was required for the establishment of the methylated imprint typical of the imprinted genes *H19* and *Gtl2-Dlk1* in the male germline (Kaneda et al., 2004).

Dnmt3B-mutants die during gestation on embryonic day 9.5, with the demethylation of minor satellite repeats (Okano et al., 1999). *Dnmt3A*$^{-/-}$, *Dnmt3B*$^{-/-}$ double homozygote ESCs, and embryos are defective in both *de novo* and maintenance methylation, and they are lethal earlier, at around embryonic day 8.5 (Okano et al., 1999). As a result of their ability to establish methylation marks, *Dnmt3A* and *Dnmt3B* are therefore the only two Dnmts classified as *de novo* methyltransferases.

Dnmt3L is homologous to *Dnmt3A* and *Dnmt3B,* but it lacks critical residues in the catalytic motifs, and it does not display methyltransferase activity. Nevertheless, *Dnmt3L* is required in both male and female germ cells to establish proper methylation patterns (Table 5.1; Bourc'his and Bestor, 2004; Bourc'his et al., 2001). *Dnmt3L*$^{-/-}$ mice are viable, but homozygotes are functionally sterile (Bourc'his et al., 2001). *Dnmt3L*$^{-/-}$ females can give rise to heterozygote embryos, but these die around embryonic day 9.5, and they lack the methylation that is characteristic of maternally silenced imprinted genes. Thus, imprinted genes that are normally expressed from the paternal allele are biallelically expressed. Paternal methylation imprints in these embryos appear to be properly maintained, which indicates that the imprint defect arises in the maternal germline and not during embryogenesis (Bourc'his et al., 2001). Although *Dnmt3L*$^{-/-}$ female germ cells are compromised in the establishment of methylation imprints, germ cells of *Dnmt3L*$^{-/-}$ males lack the methylation of transposons, and they express high levels of LINE-1 and IAP transposon RNAs (Bourc'his and Bestor, 2004). *Dnmt3L* is thought to affect methylation by upregulating the activity of *Dnmt3A* (Goll and Bestor, 2005).

Two mechanisms have been proposed to explain gene silencing by DNA methylation (Goll and Bestor, 2005). In the first case, CpG methylation may prevent the binding of transcriptional factors to their cognate DNA sequences. Alternatively—and perhaps concurrently—a class of proteins that bind methylated DNA may inhibit transcription by recruiting proteins that modify histones, which in turn reconfigure chromatin into a transcriptionally inhibitory state.

In humans, *DNMT3B* is found mutated in a rare autosomal disorder known as ICF (immunodeficiency, centromere instability, and facial anomalies) syndrome (Xu et al., 1999). ICF syndrome is associated with a loss of methylation of satellite DNA at the pericentromeric regions of chromosomes 1, 9, and 16. The demethylated chromosomes are highly prone to gains and losses of chromosome arms, and these cytogenetic abnormalities are ultimately the cause of the various clinical features associated with ICF. Mutations in genes that encode proteins that bind methylated DNA, which are thought to mediate the effects of methylated cytosines, also cause phenotypic abnormalities. For example, mutations in the *MECP2* gene cause a neurologic disorder called Rett syndrome in humans and a related disorder in mice, and mice null for *Mbd2* exhibit behavioral defects, including the compromised nurturing of pups (Goll and Bestor, 2005).

B. Histone Modifications

Although DNA methylation is clearly required to establish and maintain proper imprinted gene expression and silence transposons, it appears to be dispensable for the silencing of the imprinted X-chromosome as well as for that of a vast majority of genes, including those that contain CpG islands. In addition to

DNA, histones can also be covalently modified (Nightingale et al., 2006). Catalyzed by different families of proteins, these modifications include the acetylation, methylation, phosphorylation, polyribosylation, sumoylation, and ubiquitination of specific amino acids within the tails of various histone proteins (see the Recommended Resources section). Although the purpose of many of these modifications is currently unclear, certain modifications are correlated with transcriptionally active (i.e., euchromatin) or inactive (i.e., heterochromatin) domains. For example, in mammals, the acetylation of histone H3 lysines 4, 36, and 72 is often enriched in euchromatin, whereas the methylation of lysines 9 and 27 of histone H3 and of lysine 20 of histone H4 are associated with heterochromatin. Thus, although some modifications are mutually exclusive, accumulating evidence indicates that others act combinatorially to affect gene expression (Turner, 2002).

Although many histone modifications—along with their effects on transcription—have been described, precisely how they regulate gene expression is not known for most (Nightingale et al., 2006). Whereas some models propose that the modified histones recruit proteins and complexes that physically reconfigure chromatin into transcriptionally permissive euchromatin or transcriptionally inert heterochromatin, others contend that the histone marks regulate transcription by sterically facilitating or hindering access to the basal transcriptional machinery. It is also presently unclear how proteins or complexes that catalyze the various histone modifications are targeted to chromatin to establish a particular epigenetic state. Proposed models implicate the recruitment of histone modifiers by the transcription of noncoding RNAs, the local chromatin state, the presence or absence of other DNA-binding factors, and—not surprisingly—the DNA sequence itself (Lachner and Jenuwein, 2002). After they have been enacted, histone modifications must be reestablished during DNA replication for the faithful transmission of the epigenetic information to daughter cells. A mechanism described for the propagation of the histone mark mediated by the Suv39h methyltransferases is an elegant solution to this problem. Suv39h1 and Suv29h2 enact histone H3–lysine 9 (H3–K9) methylation, primarily at pericentromeric heterochromatin, and the loss of this modification results in chromosome segregation defects and genomic instability in mice (Peters et al., 2003). Methylated H3–K9 is bound by heterochromatin protein 1 (HP1), which in turn recruits additional Suv39 proteins, leading to the spread of H3–K9 methylation (Bannister et al., 2001). Similarly, unmodified histones incorporated during DNA replication can be modified akin to nearby histones. However, it remains to be demonstrated how the H3–K9 methylation is prevented from spreading into euchromatic regions. The answer may lie in the functions of heterochromatin-counteracting histone modifications. In fact, H3–K9 methylation is antagonized by phosphorylation of the adjacent serine (S10) and the acetylation of lysine at position 14, both of which are associated with active transcription. By the actions of self-reinforcing as well as opposing combinations of histone marks, individual chromatin signatures may be propagated and restricted at the same time.

In addition to the interplay among histone modifications, histone modifications and DNA methylation also influence each other. For example, DNMTs interact with a number of histone modifiers, which may then perform the gene-silencing function (Fuks, 2005). The EZH2 methyltransferase, which is a member of the Polycomb group (PcG) (discussed below), interacts with a

number of DNMTs both *in vitro* and *in vivo* and recruits these to EZH2 target sites; moreover, a reduction in either results in a derepression of the tested EZH2 target genes (Vire et al., 2006). DNMTs themselves are also thought to recruit other chromatin modifiers, including PcGs (Fuks, 2005). Nevertheless, it is clear that histone methylation and DNA methylation do not always appear together. For instance, whereas DNA methylation does not function in imprinted XCI, PcG-mediated histone methylation maintains imprinted XCI; conversely, although PcGs are dispensable for random XCI, DNMTs are required to maintain random XCI (Kalantry and Magnuson, 2006; Kalantry et al., 2006; Sado et al., 2000). Moreover, the proper regulation of some imprinted gene expression in mouse extra-embryonic tissues requires PcG-mediated histone methylation but not DNA methylation (Lewis et al., 2004; Umlauf et al., 2004). When found together, DNA methylation is thought to reinforce the chromatin state established by histone modifications (Klose and Bird, 2006). DNA methylation has also been proposed to be a beacon for histone modifiers after DNA replication, a mechanism that ensures that histone modifications are propagated through cell division (Sarraf and Stancheva, 2004).

In contrast with changes in the DNA code, epigenetic marks are not permanent; thus, the chromatin modifications must be reversible. Whereas proteins that reverse DNA methylation have not been purified, several families of proteins that catalyze the removal of histone modifications have recently been described. Prominent examples include histone deacetylases and histone demethylases (Holbert and Marmorstein, 2005; Trojer and Reinberg, 2006).

Mouse mutations resulting in a lack of proteins that catalyze (or help catalyze) histone modifications clearly demonstrate their importance during development (Table 5.1). That DNA and histone modifications are essential for proper development and have the ability to influence transcription, however, does not necessarily define them as epigenetic mediators of cellular memory. Many proteins that do not chemically modify chromatin are capable of modulating transcription and, in turn, of affecting cell fate and development. For the chromatin modifications to represent a true heritable epigenetic code, they must have predictive value. For example, cells that are subject to a change in these modifications should not only change their own transcriptional profile, but they should also stably transmit the altered expression pattern to their progeny. The evolutionarily conserved PcG proteins represent the most prominent example of chromatin modifiers that have functions that maintain cellular memory.

V. EPIGENETIC REGULATION BY THE POLYCOMB GROUP

Proper anterior–posterior body segmentation requires the function of homeotic (Hox) genes (Grimaud et al., 2006). Hox genes were first identified through mutations in *Drosophila* that resulted in the transformation of body segments. The appropriate expression of the Hox genes is triggered as a result of the graded expression of various transcription factors and morphogens. These factors are only transiently expressed; however, after they are established, Hox gene expression patterns are maintained throughout development. Genetic screens were therefore performed to identify genes that propagated patterns of Hox gene expression. Two classes of genes were discovered in

the screens: the PcG and the trithorax group (trxG). From these and subsequent genetic experiments, PcGs were inferred to repress Hox genes, whereas trxGs antagonized PcGs to maintain active states of expression.

The founding PcG member is, as the name suggests, *Polycomb* (*Pc*). *Pc* was identified as a dominant mutation in *Drosophila* that caused the ectopic formation of sex combs on the legs of male flies (Grimaud et al., 2006). Homozygous loss-of-function *Pc* mutations, on the other hand, led to the transformation of embryonic segments into the posteriormost segment. The mutant phenotypes suggested the ectopic expression of Hox genes of the bithorax complex, and the finding that *Pc* mapped outside of the bithorax complex indicated that it functioned as a negative regulator of the homeotic genes in *trans*. Subsequently, genetic screens identified a group of genes that had mutations that either enhanced or recapitulated the phenotypes of mutant *Pc* alleles (Grimaud et al., 2006).

The cloning of the first PcG gene, *Pc*, offered an immediate potential molecular explanation of PcG function (Grimaud et al., 2006). *Pc* shares homology with HP1 in a region called the *chromodomain*. HP1 is an evolutionarily conserved nonhistone protein that is implicated in heterochromatin packaging via the chromodomain. The cloning of other PcG genes soon thereafter resulted in the identification of another conserved motif, the SET domain, which is found in diverse proteins (including in some trxG proteins) and which also affects chromatin structure. PcGs were subsequently shown to biochemically function in multimeric complexes to modify histones via the SET domain.

PcGs exist in at least two distinct complexes. The Polycomb repressive complex 2 (PRC2) in mammals, which consists of the proteins EED, EZH2, SU(Z)12, RBAP46, and RBAP48, methylates lysine 27 of histone H3 (H3–3mK27) both *in vivo* and *in vitro* (Grimaud et al., 2006). H3–3mK27 in turn provides a substrate for the assembly of Polycomb repressive complex 1 (PRC1) via binding of the Pc protein through the chromodomain. PRC1 is thought to then compact chromatin in a transcriptionally repressed state. The core components of *Drosophila* PRC1, which consist of Psc, Ph, Pc, and dRing, have been shown to physically compact nucleosomal arrays and inhibit their remodeling and transcription *in vitro* (Marx, 2005). This chromatin compaction model of PcG function, however, has been contested by findings *in vivo* in which PcGs repress transcription by preventing RNA polymerase from initiating transcription rather than by sterically hindering its access to chromatin (Marx, 2005).

In mammals, the best evidence for the role of PcGs in cellular memory is during XCI. In mice, XCI occurs in two distinct forms: imprinted and random. Imprinted XCI results in preferential silencing of the paternal X chromosome (Xp), initially in all cells of the early embryo (Heard, 2005). Subsequently, during the late blastocyst stage, the imprint is erased only in the cells that are destined to give rise to the embryo proper (the inner cell mass or the epiblast). Later in development, these cells randomly inactivate either the maternal or the paternal X chromosome. Extraembryonic cells (the trophectoderm and primitive endoderm lineages), on the other hand, maintain imprinted XCI. In both cases, the same X chromosome remains inactive in all descendants of progenitor cells in which it is first silenced; XCI therefore provides a potent model system to investigate heritable forms of transcriptional regulation.

VI. EPIGENETIC CONTROL OF X-CHROMOSOME INACTIVATION

XCI is characterized by an ordered series of epigenetic events (Figure 5.2). Both imprinted and random XCI are prefaced by the expression of the X-linked nonprotein coding *X (inactive)-specific transcript* (*Xist*) RNA from the prospective Xi (Heard, 2005). *Xist* transcription in *cis* is required to initiate silencing along the X chromosome. During imprinted XCI, *Xist* is expressed as early as the two-cell stage, and the RNA visibly begins to coat the Xp at the four-cell stage, preceding the transcriptional silencing of X-linked genes. The progressive spreading of *Xist* along the Xp correlates with the gradual silencing of genes on either side of the *Xist* locus, both of which are largely complete by the blastocyst stage.

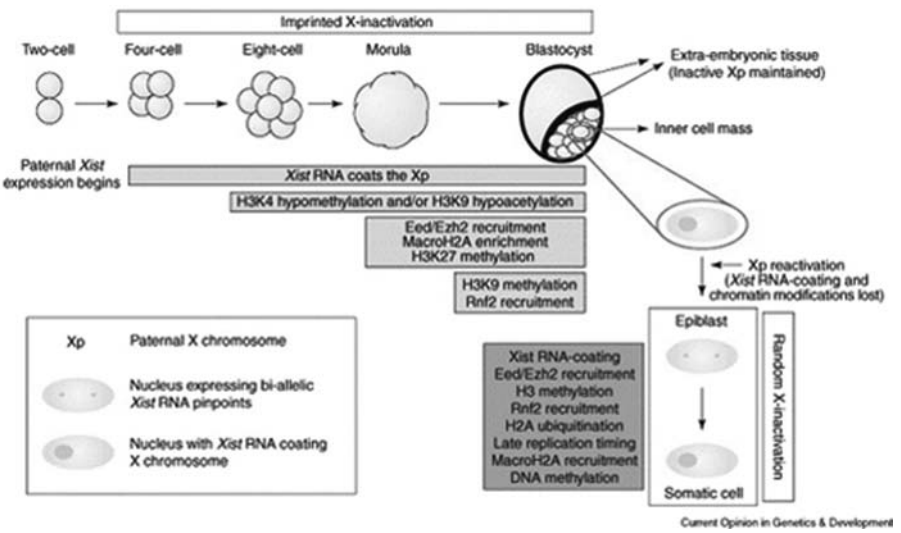

FIGURE 5.2 **The epigenetics of X-chromosome inactivation (XCI) during preimplantation development**. XCI during embryogenesis occurs twice and is presaged by the expression of the *Xist* RNA. The first wave of XCI results in the preferential inactivation of the paternal X chromosome (Xp), which is referred to as *imprinted XCI*. At the late two-cell stage, the Xp begins to express the *Xist* RNA. At the four-cell stage, *Xist* RNA starts to coat the Xp (Huynh and Lee, 2003; Okamoto et al., 2004). Subsequently, a series of chromatin modifications characterizes the inactive X chromosome (Xi), and this is concomitant with the initiation of the transcriptional silencing of genes along the Xp (Costanzi et al., 2000; de Napoles et al., 2004; Mak et al., 2004; Okamoto et al., 2004; Silva et al., 2003). The Xi becomes hypomethylated at lysine 4 and hypoacetylated at lysine 9 of histone H3 at the eight-cell stage, when the silencing of X-linked genes is first detected (Huynh and Lee, 2003; Okamoto et al., 2005) Then, at the morula stage, the following are enriched on the Xi: the Polycomb group proteins EED and EZH2; the histone modification that they mediate, trimethylation of lysine 27 of histone H3 (H3–3 mK27); and the histone variant macro H2A. It should be noted that Polycomb group enrichment on the Xp occurs during the window that XCI initiates, because the spread of gene silencing along the Xp may not be complete until the blastocyst stage (Huynh and Lee, 2003). At the late blastocyst stage, cells of the inner cell mass, which will give rise to the embryo proper, reverse imprinted X inactivation (Mak et al., 2004; Okamoto et al., 2004). *Xist* RNA coating and the chromatin modifications are lost from the Xp. These cells then undergo random X inactivation, which results in the silencing of either the maternal or the paternal X chromosome shortly after implantation (Plath et al., 2002). *Light gray* boxes indicate the approximate timeframe of the appearance of proteins or posttranslational histone modifications on the X chromosome. Epigenetic changes indicated in the *dark gray* box are listed in the order of appearance during random X inactivation. (From Nusinow and Panning, 2005.)

PcGs have also been implicated in the initiation of XCI. PRC2 proteins and H3–3mK27 accumulate on the Xi soon after or coincident with *Xist* RNA coating during both imprinted and random XCI, which correlates with the spreading of silencing along the Xi (Heard, 2005). Moreover, the misexpression of *Xist* results in the concomitant accumulation of PRC2 proteins and H3–3mK27 (Plath et al., 2003; Silva et al., 2003). Furthermore, in the embryo as well as in ESCs and trophoblast stem cells (TSCs), PRC2 and PRC1 proteins—along with the histone modifications they catalyze—are mostly found enriched on the Xi early during differentiation (Heard, 2005). ESCs and TSCs are model cell types for the study of random and imprinted XCI, respectively (Plath et al., 2003; Silva et al., 2003). The temporal pattern of the accumulation of PcGs on the Xi coincides with the gradual silencing of the X-linked genes, and it has led to the suggestion that the PcGs contribute to the initiation and/or establishment of XCI (Plath et al., 2003; Silva et al., 2003).

A role for PcGs in XCI was first suggested by the reactivation of the Xi in embryos mutant for the mouse PcG gene *Embryonic ectoderm development* (*Eed*) (Wang et al., 2001). In $Eed^{-/-}$ mutant females, XCI is initiated but not properly maintained (Kalantry et al., 2006; Wang et al., 2001). Subsequently, $Eed^{-/-}$ embryos (both male and female) were also shown to harbor defects in the silencing of some imprinted genes (Mager et al., 2003). EED is an essential noncatalytic component of the PRC2 complex; when it is mutated, the entire complex is disabled, and histone methylation catalyzed by the PRC2 complex is lost (Montgomery et al., 2005). The exact biochemical role of EED is unclear, but its WD-40 motifs are thought to mediate protein–protein interactions. Importantly, EED is the only mouse PcG gene described to date to be required for XCI; mouse mutations in other PRC2 components exist, but they have not been analyzed for XCI defects. The absence of EED leads to the severe downregulation of both the core PRC2 components (EZH2 and SUZ12) and the PRC2-mediated histone methylation (Montgomery et al., 2005).

Although EED is preferentially enriched on the Xi early in XCI, it is the differentiated extra-embryonic trophoblast cells that normally do not enrich EED on the Xi in wild-type embryos, which exhibit the reactivation of the previously silenced Xp in $Eed^{-/-}$ embryos (Kalantry and Magnuson, 2006; Kalantry et al., 2006; Wang et al., 2001). This observation raised the question of how the transient enrichment of *Eed* on the Xi in extra-embryonic progenitor cells contributes to the stable transcriptional silencing of the Xi in differentiated cells derived from these progenitors.

To resolve this apparent paradox, $Eed^{-/-}$ TSCs were derived and analyzed for XCI defects (Kalantry et al., 2006). TSCs originate from the extra-embryonic trophectoderm and exhibit the exclusive inactivation of the Xp as a result of imprinted XCI. The Xi in $Eed^{-/-}$ TSCs and in cells of the trophectoderm-derived extra-embryonic ectoderm in $Eed^{-/-}$ embryos was found to remain transcriptionally silent, despite lacking the PcG-mediated histone modifications (and all other known Xi factors, including *Xist* RNA coating) that normally characterize the Xi heterochromatin (Kalantry et al., 2006). Although undifferentiated $Eed^{-/-}$ TSCs maintained XCI, reactivation of the Xi occurred when these cells were differentiated (Figure 5.3). These results indicate that PcG complexes are not necessary to maintain the transcriptional silencing of the Xi in undifferentiated stem cells.

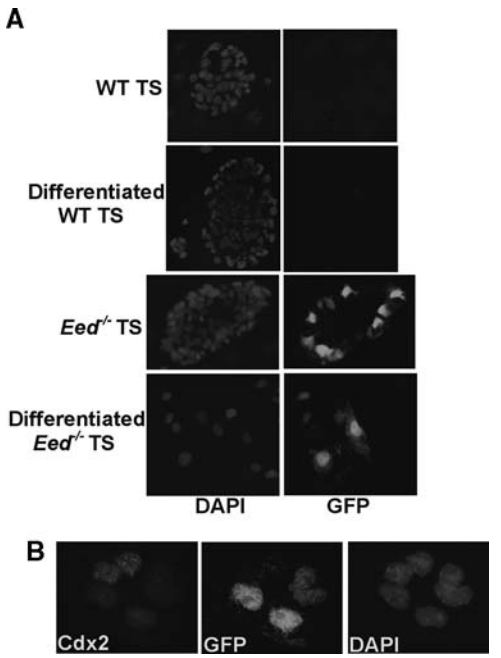

FIGURE 5.3 **Imprinted X chromosome inactivation defects in differentiating *Eed*$^{-/-}$ trophoblast stem cells (TSCs).** A paternal X-linked *GFP* transgene (*Xp-GFP*), which faithfully recapitulates endogenous X-linked gene activity, is used to monitor Xp-linked gene activity. Nuclei are stained with 4,6-diamidino-2-phenylindole dihydrochloride. **A,** Wild-type TSCs undergo imprinted X-chromosome inactivation of the Xp as indicated by a complete lack of *Xp-GFP* expression in all cells, including after differentiation. *Eed*$^{-/-}$ TSC colonies contain cells with an active Xp; these cells are located preferentially at the periphery of the colonies, where differentiated cells are found. **B,** Immunofluorescence detection of CDX2, a marker of undifferentiated trophectoderm cells, in cultured *Eed*$^{-/-}$ TSCs. CDX2 is downregulated in mutant cells harboring an active Xp as indicated by *GFP* expression, which demonstrates that these cells are differentiated. (From Kalantry et al., 2006. See color insert.)

In addition to the trophectoderm, a second extra-embryonic lineage, the primitive endoderm (PE) lineage, also undergoes imprinted XCI (Heard, 2005). Cell lines typical of this lineage have also been derived and shown to exclusively inactivate the Xp (Kalantry et al., 2006). Similar to that seen in the trophectoderm and epiblast lineages (and in the TSC and ESC lines representing these lineages), PcGs are only transiently enriched on the Xi in the PE and its derivatives. After the PE differentiates into the parietal and visceral endoderm cell layers, which occurs soon after the PE specification, the Xi-accumulation of PcGs is no longer apparent. In *Eed*$^{-/-}$ embryos, the Xi in the PE derivatives is silenced properly, and it does not become reactivated (Kalantry and Magnuson, 2006; Kalantry et al., 2006). Primitive endoderm originates from the inner cell mass at the late blastocyst stage, before the erasure of the imprinted silencing of the Xp, and it maintains the imprinted XCI of the Xp established earlier (Kalantry and Magnuson, 2006; Kalantry et al., 2006). *Eed*$^{-/-}$ embryos may therefore properly initiate silencing in this lineage as a result of the persistence of maternal EED protein. The PE also differentiates during the timeframe when maternal EED protein is last seen; in addition, although PcGs are normally not enriched on the Xi in the

differentiated PE derivatives, EED is dispensable for the maintenance of XCI in parietal and visceral endoderm, thus providing an explanation for the lack of Xi-reactivation defects in these cells in *Eed*$^{-/-}$ embryos.

Together, these studies argue that cells that are stably maintaining their differentiation state and thus their transcriptional profile (i.e., undifferentiated or fully differentiated cells) may not need PcG proteins to propagate transcriptional silencing. Thus, PRC2 and the associated histone modifications on the Xi may function to block alterations in chromatin structure that promote transcriptional activation rather than to stabilize the heterochromatin per se in undifferentiated cells. Therefore, PcGs appear to maintain cellular memory by preventing the transcriptional activation of heterochromatin during chromatin reconfiguration that, for example, accompanies differentiation. This mechanism of action of PcGs may also explain the requirement in *Drosophila* of the *Eed* homolog ESC early in development for the maintenance of gene silencing later (Ringrose and Paro, 2004).

The analysis of *Eed*$^{-/-}$ TSCs and embryos, however, does not address whether PcGs are required to initiate XCI. *Eed*$^{-/-}$ TSCs are derived from blastocyst-stage embryos, which harbor considerable amounts of maternally derived EED protein (Kalantry and Magnuson, 2006; Kalantry et al., 2006; Shumacher et al., 1996). Whereas zygotic *Eed* transcription is first evident at embryonic day 5.5, EED protein is detected on the Xi as late as embryonic day 3.5. Thus, all EED protein detected in preimplantation embryos is maternally derived, and it may suffice to initiate imprinted XCI, even in *Eed*$^{-/-}$ embryos. However, the maternal protein was hypothesized to be depleted by the time random XCI initiates during postimplantation development (Kalantry and Magnuson, 2006). It would therefore be possible to assess whether EED—and, therefore, PRC2—is required to initiate random XCI.

To define the time at which maternally derived EED protein becomes depleted, peri-implantation–stage embryos generated from an *Eed*$^{+/-}$ intercross were analyzed (Kalantry and Magnuson, 2006). The sire also carried on its X chromosome a *GFP* transgene. The X-linked *GFP* transgene is exclusively transmitted to female embryos, and it is expressed before XCI-mediated silencing (Wang et al., 2001). Thus, this transgene facilitates the identification of female embryos and the analysis of paternal X-chromosome activity. Embryonic day 4.5 embryos were stained by immunofluorescence for H3–3mK27, the histone H3 methylation mediated by the EED-containing PRC2 complex. A majority of these embryos showed prominent accumulation of H3–3mK27 in the trophectodermal cells (Figure 5.4, *A*). Cells of the epiblast, which is derived from the inner cell mass, lack the Xi enrichment of H3–3mK27, which indicates that these cells have yet to undergo random XCI. During random XCI, H3–3mK27 appears on the Xi concomitant with *Xist* RNA coating during the initiation phase (Plath et al., 2003; Silva et al., 2003). The epiblasts fluoresce green as a result of the expression of the paternal X-linked *GFP* transgene, which reflects the reactivation of the Xp before random XCI. A proportion of embryonic day 4.5 embryos, however, did not show noticeable H3–3mK27 staining in any cell of the embryo, thereby indicating that they are *Eed*$^{-/-}$ (Figure 5.4, B; Kalantry and Magnuson, 2006). These results therefore indicate that maternal EED protein is depleted by embryonic day 4.5.

However, the epiblast or epiblast derivatives of wild-type postimplantation embryos at embryonic days 5.5, 6.5, and 7.5 all showed Xi accumulation of H3–3mK27 or EED and *Xist* RNA, signifying that random XCI

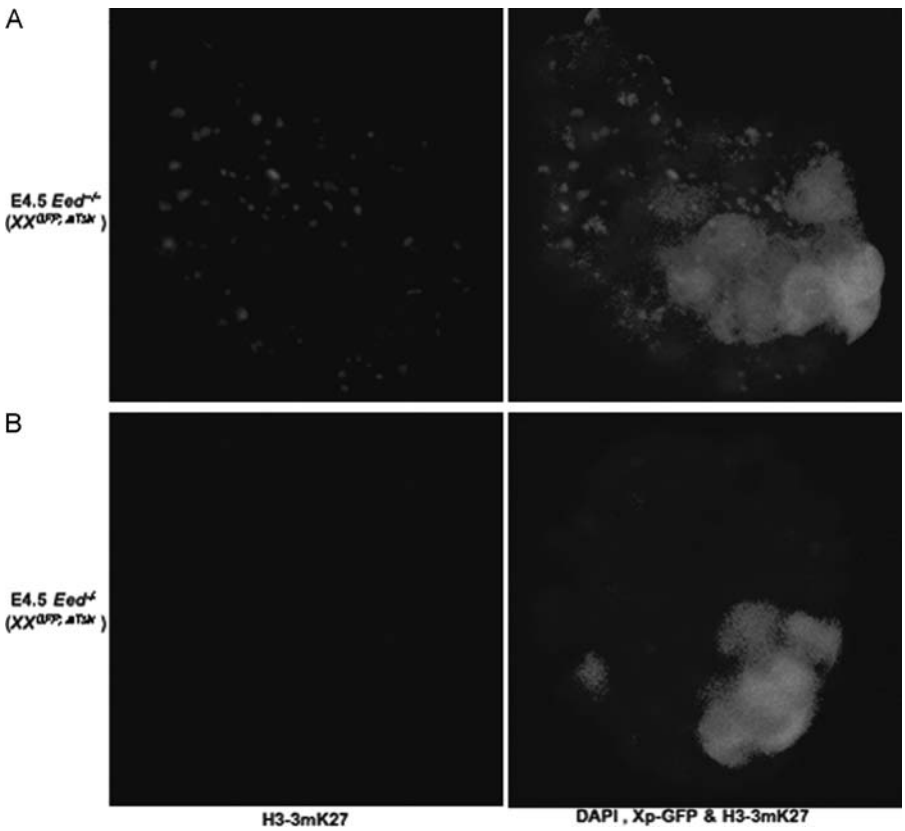

FIGURE 5.4 **The absence of EED activity before the initiation of random X-chromosome inactivation (XCI) in *Eed*$^{-/-}$ embryos.** Immunofluorescence detection of histone H3–trimethyl lysine 27 (H3–3 mK27). **A,** Readout of EED activity in a wild-type embryonic day 4.5 embryo shows H3–3 mK27 enrichment on the inactive X chromosome in all trophectodermal cells but which is largely absent in the inner cell mass cells, thus indicating that these cells have not undergone random XCI. The inner cell mass cells selectively express the paternal X-linked *GFP* transgene (*Xp-GFP*) as a result of the erasure of the imprint that ensures the imprinted XCI of the Xp. Whereas the inner cell mass has erased the imprint, the trophectoderm has stably maintained imprinted XCI and hence is negative for *Xp-GFP.* Staining with 4,6-diamidino-2-phenylindole dihydrochloride detects nuclei in blue. **B,** Immunofluorescence staining showing a lack of histone H3–3 mK27 accumulation on the inactive X chromosome in all cells at embryonic day 4.5, indicating that EED activity is absent in mutant embryos before the initiation of random XCI. (From Kalantry and Magnuson, 2006. See color insert.)

has initiated in these cells (Figure 5.5, *A*; Kalantry and Magnuson, 2006). *Eed*$^{-/-}$ embryos at these stages, expectedly, lacked all H3–3mK27 or EED (Figure 5.5, *B*). Despite the absence of EED and/or H3–3mK27 in these embryos, when random XCI initiates, random XCI occurrs properly (Kalantry and Magnuson, 2006). These results, therefore, are evidence that PcGs are dispensable for the initiation of random XCI. PcGs are also not essential for the maintenance of random XCI, which is in contrast with their requirement during imprinted XCI maintenance.

Although clearly dispensable for random XCI initiation, a requirement for PcGs in imprinted XCI initiation cannot formally be ruled out. The timing and order of epigenetic modifications that characterize the Xi during imprinted and random XCI are largely the same, but differences do exist.

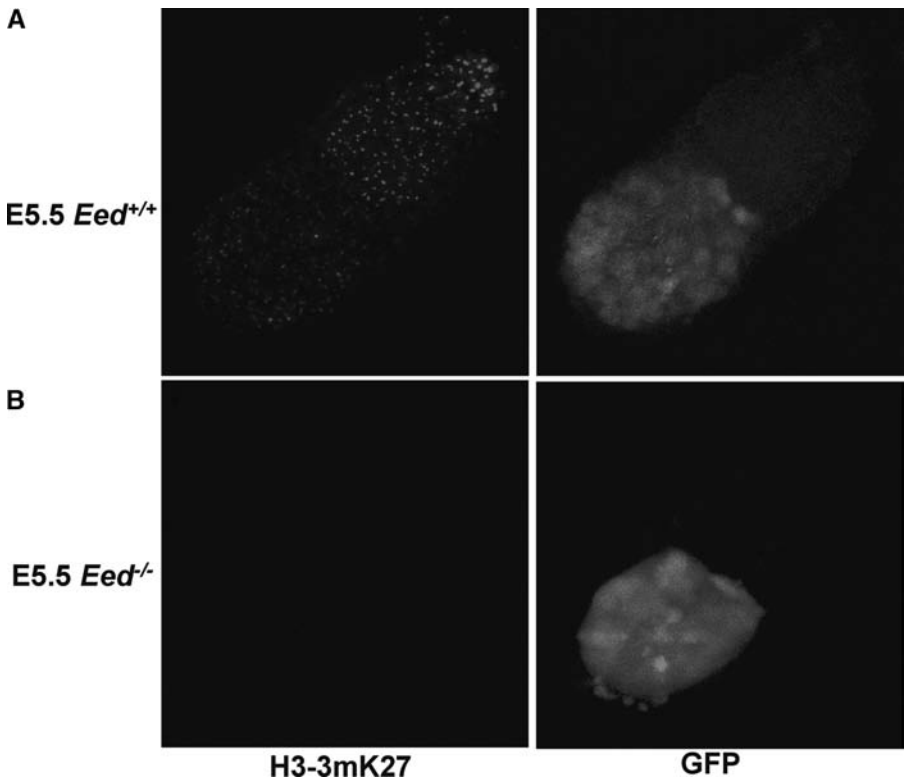

FIGURE 5.5 **The enrichment of histone H3–trimethyl lysine 27 (H3–3mK27) on the inactive X during the initiation of random X-chromosome inactivation (XCI). A,** Immunofluorescence detection in a wild-type embryonic day 5.5 embryo showing inactive X accumulation of H3–3mK27 in all cells of both the extra-embryonic and embryonic lineages (i.e., the trophectoderm-derived extra-embryonic ectoderm and the epiblast, which undergo imprinted and random XCI, respectively). The epiblast cells fluoresce green as a result of the expression of the X-linked *GFP* transgene. **B,** $Eed^{-/-}$ embryos lack all H3–3mK27 staining. Despite the absence of EED activity before and during random XCI initiation, however, random XCI is unaffected. (From Kalantry and Magnuson, 2006. See color insert.)

For example, DNA methylation is required to maintain random XCI but not imprinted XCI. Thus, although PcGs are not necessary to initiate random XCI, they may be indispensable for imprinted XCI initiation. To date, however, proteins or protein complexes that contribute to the initiation of epigenetic transcriptional silencing have not been definitively identified.

In sum, recent progress in many model systems has elucidated an array of covalent modifications of histones. Along with the previously identified methylation of cytosines in DNA, these modifications are proposed as transgenerational modulators of transcriptional states, because they correlate with active or silenced gene expression. These marks are hypothesized to maintain cellular memory by establishing and maintaining transcriptional patterns by affecting chromatin conformation. It remains to be determined, however, precisely how most of these modifications are targeted to specific regions of the genome and how they bring about changes in chromatin structure and gene expression. In addition, only a few of these marks have been shown to propagate transcriptional states through cell division.

Nevertheless, it is becoming increasingly clear that the proteins or complexes that enact or read these modifications play critical roles in modulating the developmental potential of cells, and they are proving to be dysregulated in disease. For example, cell types with stem cell or progenitor cell character display reduced levels of global histone H3–lysine 9 trimethylation (H3–3mK9) and histone H4–lysine 20 trimethylation (H4–3mK20) but high levels of histone acetylation (Baxter et al., 2004). The same pattern is observed in human cancers (increased levels of H4–3mK20 and diminished histone acetylation), buttressing the notion that tumorigenic cells resemble an undifferentiated state (Fraga et al., 2005). Similarly, the Polycomb group has been implicated in both the maintenance of embryonic stem cell pluripotency and the proliferative capacity of various cancers (Buszczak and Spradling, 2006; Valk-Lingbeek et al., 2004). Specific combinations of chromatin modifications thus appear to be critical in determining the level of cell differentiation, both during embryonic development and in diseases such as cancers. It comes as no surprise, then, that chromatin modifiers have become attractive targets for cancer therapy and for modulating the developmental potential of cells (Jenuwein, 2006). Clearly, the study of epigenetic transcriptional regulation is only just beginning to yield critical insight into development and disease.

SUMMARY

- Various epigenetic markings of chromatin components—the reversible, covalent modifications of DNA and histones—are correlated with active and repressed transcriptional states.
- DNA and histone modifications are hypothesized to modulate chromatin conformation.
- The targeting of chromatin modifiers to specific regions of the genome as well as the mechanisms by which most chromatin modifications affect chromatin structure are presently unclear.
- DNA and histone modifications are, however, instrumental in maintaining cellular identity during embryonic development, and they are increasingly being found to be dysregulated in disease.

GLOSSARY

Cellular memory
The epigenetic maintenance of cellular identity via the propagation of gene expression profiles across cell division.

DNA methylation
The epigenetic modification primarily of cytosines in cytosine–guanine dinucleotides catalyzed by a family of DNA methyltransferase proteins.

Epigenetic inheritance
All transmitted cellular material other than DNA; commonly defined as reversible covalent modifications of chromatin components (i.e., DNA and histones) that regulate gene transcription.

Histone modifications
The regulation of transcription via a combination of the acetylation, methylation, phosphorylation, polyribosylation, sumoylation, and ubiquitination of specific histone amino acids, which are catalyzed by different families of proteins.

Imprinting
The parent of the origin-specific expression of genes, which results in transcription of either the maternal or paternal allele but not both.

Polycomb group
A family of proteins that maintains cellular memory by repressing gene transcription via the epigenetic modifications of histones.

X-chromosome inactivation
The epigenetic transcriptional silencing of genes on one of the two X chromosomes in female mammals, resulting in the equivalence of X-linked gene expression between males and females.

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RECOMMENDED RESOURCES

A catalog of mouse and human imprinted genes, along with their known functions, can be found at http://igc.otago.ac.nzandhttp://www.mgu.har.mrc.ac.uk.

A detailed depiction of the various histone modifications can be found at http://www.histone.com/modification_map.htm.

6 NEW INSIGHTS INTO VERTEBRATE ORIGINS

BILLIE J. SWALLA

Center for Developmental Biology and Biology Department, University of Washington, Seattle, WA; Friday Harbor Laboratories, Friday Harbor, WA; and the Smithsonian Marine Station, Fort Pierce, FL

INTRODUCTION

A. History of Hypotheses of Chordate Origins

Vertebrates share several distinct morphologic characteristics with three invertebrate groups: lancelets, tunicates, and hemichordates (Figure 6.1). Tunicates, lancelets, and vertebrates have traditionally been considered to be a monophyletic group—the chordates—that shares five morphologic features: a notochord, a dorsal neural tube, an endostyle, a muscular postanal tail, and pharyngeal gill slits. Hemichordates share some of these chordate features: the pharyngeal gill slits (Figure 6.2), an endostyle, and a postanal tail. Previously, hemichordates were thought to contain a notochord homolog (the stomochord) and a dorsal neural tube in the neck region, but recent evidence from developmental genetics has questioned these homologies. Developmental genetics and genomics have allowed for the reexamination of the question of chordate origins by comparing developmental gene expression in embryos of different phyla. This powerful approach has allowed new insights into the molecular mechanisms underlying morphologic changes. Genomics has allowed for investigations into the phylogenetic relationships of the chordates and their invertebrate relatives, and for the comparison of the shared genetic pathways in related embryos. We review current research on this topic and show that our view of the chordate ancestor has changed during the past 10 years. For years, the chordate ancestor has been considered to be a filter-feeding, tunicate-like animal with a tiny chordate tadpole larva. However, recent evidence from my laboratory and others has shown that the chordate ancestor was more likely a benthic worm with a mouth and pharyngeal gill slits supported by cartilaginous gill bars (Cameron et al., 2000; Gerhart et al., 2005; Rychel et al., 2006;

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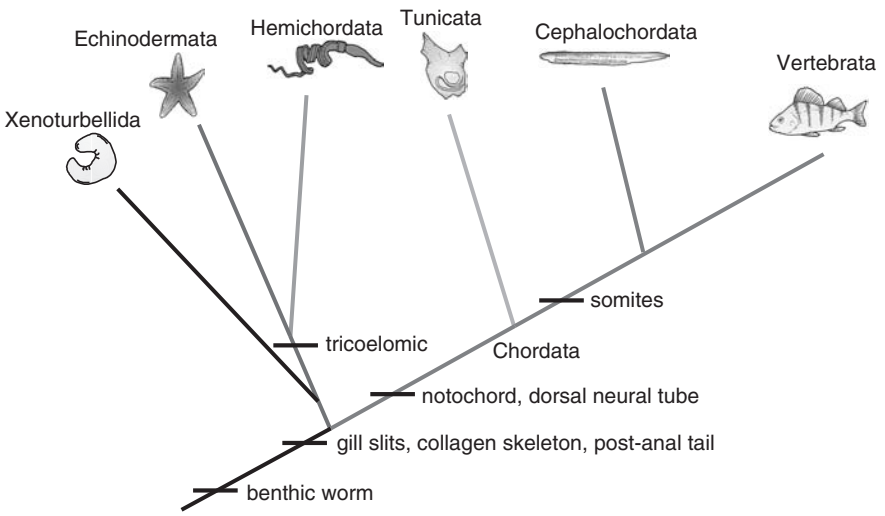

FIGURE 6.1 **Deuterostome phylogeny.** There are five distinct adult body plans seen among the deuterostomes. Echinodermata and Hemichordata have distinctly different body plans, but similar tricoelomic feeding larvae. Xenoturbellids are a newly described deuterostome phylum, and little is known about their development. The fourth group exhibiting a distinct adult body plan is the Tunicata, which is usually considered a subphylum within Chordata. The last groups are the Cephalochordata and the Vertebrata, which are considered Chordata subphyla with the tunicates. Morphologic characters that are shared between major groups are marked on the figure. Our evidence suggests that the chordate ancestor was a benthic worm that had gill slits, a collagenous skeleton, and a postanal tail. Somites are a developmental feature that unites Cephalochordata and the Vertebrata. (Redrawn with modification from Rychel et al., 2006.)

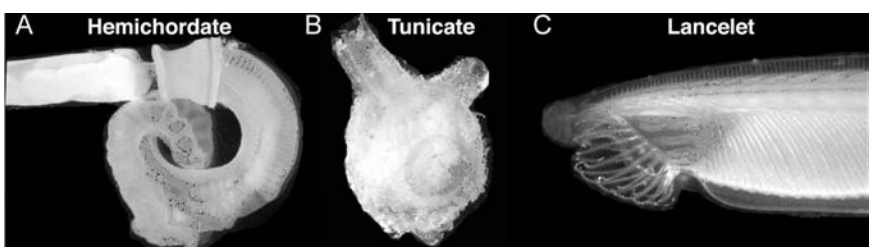

FIGURE 6.2 **Three distinct invertebrate body plans. A,** An adult enteropneust hemichordate, *Saccoglossus kowalevskii*; **B,** a tunicate molgulid ascidian; and **C,** a lancelet, *Branchiostoma virginae*, showing the dramatic differences in their adult body plans. **A,** The mouth of the enteropneust hemichordate is hidden in the collar region, directly behind the anterior proboscis. **B,** The mouth of the tunicate is moved upward at metamorphosis and shown here as the siphon to the top left, whereas the anus empties into the buccal siphon, shown to the top right. **C,** The lancelet mouth has been modified for filter feeding, as shown by the cirri at the anterior, ventral side, to the left. (All animals were collected and photographed at the Smithsonian Marine Station at Fort Pierce, Florida.)

Rychel and Swalla, 2007). Further research in developmental genetics and genomics is likely to be fruitful for solving some of the remaining homologies between the hemichordates and the chordates.

Three basic hypotheses of chordate origins are shown in Figure 6.3 (Garstang, 1928; Romer, 1967; Jefferies, 1986; Jollie, 1973; Gee, 1996; Gerhart et al., 2005; Rychel et al., 2006). One early scenario of chordate origins, which is still quite popular, is the view of chordate origins that was first hypothesized by Garstang near the turn of the century (Figure 6.3, A; Garstang, 1928). This theory espouses the notion that the echinoderm and

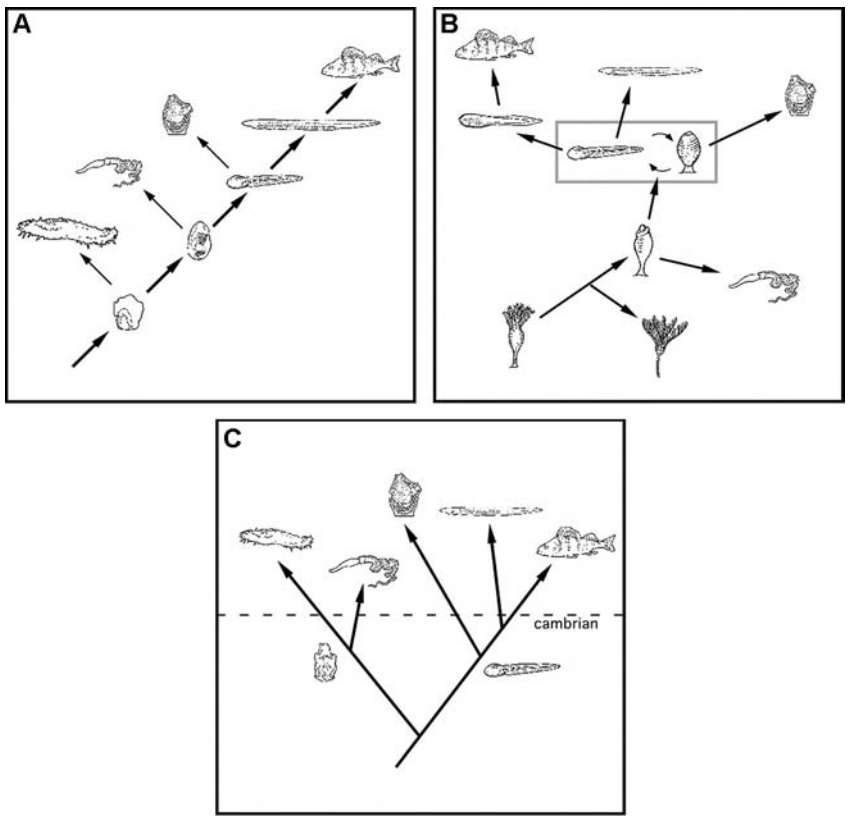

FIGURE 6.3 **Theories of chordate origins: which ones fit the available data? Several possible theories of chordate origins are depicted here. A,** Theory 1 was first proposed by Garstang, and it espouses the notion that the nonfeeding ascidian tadpole larva evolved from echinoderm-like and hemichordate-like larvae. However, developmental gene expression patterns in the different larvae show that echinoderm larvae and hemichordate tornaria larvae are very similar, but that both differ markedly from chordate expression patterns (Swalla, 2006). **B,** Theory 2 was popularized by Romer, and it depicts the deuterostomes as all evolving from a pterobranch hemichordate. Phylogenetic and fossil evidence suggest that this is an unlikely scenario, because chordates, hemichordates, and echinoderms all appear during the early Cambrian era. **C,** Theory 3 is a compilation of all available phylogenetic, fossil, and gene expression data. Parts of this theory were put first put forth by Jollie, who considered Garstang's and Romer's theories to be extremely unlikely. The molecular evidence suggests that the chordate tadpole larva had an independent origin from an ancestor with a feeding dipleurulid larva. In this scenario, the chordate body plan would have evolved *de novo* in a direct-developing soft-bodied worm-like ancestor. The notochord would have evolved from the co-opting of genes used for other functions in the ancestor. Now the search is on to determine which of the genes were most important in the evolution of this novel structure.

hemichordate larvae "evolved" into the ascidian tadpole larvae, and that the adults of echinoderms, hemichordates, and tunicates developed independently. However, as reviewed here, recent developmental data show that the features that hemichordates and chordates share are adult features rather than larval ones (Rychel et al., 2006; Swalla, 2006). These results would favor the evolution of chordates from a direct developing hemichordate (Gerhart et al., 2005; Rychel et al., 2006) rather than from hemichordate tornaria larvae. Further comments on Garstang's theory and genetic evidence against it are nicely summarized in Lacalli (2005).

Most textbooks still carry the scenario that all deuterostomes evolved from a colonial hemichordate: a pterobranch; this idea was first published by Romer in 1967 (Figure 6.3, B). This theory was popularized because the fossil record has an abundance of colonial hemichordates, called *graptolites,* and because lophophorates were thought to be related to deuterostomes (Romer, 1967; Gee, 1996). Molecular phylogenetics has shown that the lophophorates are part of a large group of animals called the *lophotrochozoa* (Halanych, 2004) and that the colonial pterobranchs are derived hemichordates (Cameron et al., 2000). Collectively, these new data bring into question the widely held view of deuterostome evolution that was popularized by Romer.

In 2000, we presented a new hypothesis based on the new molecular phylogenies and developmental gene expression patterns (Figures 6.1 and 6.3, C; Cameron et al., 2000). In this scenario, the deuterostome ancestor is worm-like, and the larvae of hemichordates and echinoderms developed independent of ascidian tadpole larvae. During the ensuing years, developmental gene-expression data have continued to favor a worm-like deuterostome ancestor (Lowe et al., 2003; Gerhart et al., 2005; Rychel et al., 2006; Delsuc et al., 2006). Developmental genomics and genetics can provide key pieces of evidence for understanding chordate origins. Genomic information will soon be available for a single member of each of the deuterostome monophyletic groups: echinoderms (the purple sea urchin *Strongylocentrotus purpuratus*), hemichordates (an acorn worm, *Saccoglossus kowalevskii*), tunicates (a solitary ascidian *Ciona intestinalis* and the pelagic appendicularian *Oikopleura dioica*), cephalochordates (a lancelet *Branchiostoma floridae*), and many vertebrate species. Initial analyses suggest that deuterostomes share many developmental genetic pathways during early embryonic and larval development (Davidson and Erwin, 2006; Swalla, 2006). Developmental genetics can be highly informative by illuminating how these similar genetic pathways are expressed in different times and places to elaborate the final morphology of the larvae and the adults (Swalla, 2006). We next review the latest findings of molecular phylogenetics and genomic analyses, examine developmental gene expression in different deuterostome phyla, and discuss the origin of the vertebrates in the light of new data published during the past 15 years.

B. Molecular Phylogenetics of Deuterostomes

Phylogenetic relationships within the deuterostomes are critical to understanding the evolutionary changes that have occurred during chordate and vertebrate evolution (Figure 6.1; Zeng and Swalla, 2005). Deuterostome phylogenetic relationships have been reviewed extensively elsewhere (Halanych, 2004; Zeng and Swalla, 2005), so they will be briefly summarized here. Schaeffer (1987) examined morphologic and phylogenetic evidence and concluded that the deuterostomes (the group of animals that contains the vertebrates) were monophyletic. Later, in 1994, two papers examined, for the first time, deuterostome relationships using 18S rDNA. Wada and Satoh (1994) showed that deuterostomes were monophyletic, presented evidence that chaetognaths were not deuterostomes, and showed that echinoderms and hemichordates were sister groups (albeit with low bootstrap support). Turbeville et al. (1994) increased the deuterostome 18S rDNA data set and used the notochord as a morphologic marker to place ascidians as chordates. Later, Cameron et al. (2000) greatly increased the number of tunicates and hemichordates in

the deuterostome 18S rDNA database and showed that echinoderm and hemichordates are sister groups with high bootstrap support with all methodologic analyses for phylogenetic reconstructions. Morphologic and molecular data since that time have continued to confirm the sister-group relationship of echinoderms and hemichordates (Halanych, 2004; Smith et al., 2004; Zeng and Swalla, 2005; Bourlat et al., 2006).

The tunicates, although monophyletic (Swalla et al., 2000), have been difficult to place within the deuterostomes with 18S and 28S combined ribosomal sequence analysis (Figure 6.1; Winchell et al., 2002). Recent genome phylogenies constructed with hundreds of genes have suggested that tunicates are more closely related to vertebrates than lancelets (Blair and Hedges, 2005; Delsuc et al., 2006; Bourlat et al., 2006). Although the phylogenetic relationship of the tunicates to the vertebrates is still in question, it is clear that the developmental programs that are activated in ascidian embryos for specific tissues are quite similar to those seen in vertebrate development (Passamaneck and Di Gregorio, 2005; Swalla, 2004; 2006). Ascidians have a number of important transcription factors localized in the egg cytoplasm that are necessary for some tissue development, so they have been described as having mosaic development (Swalla, 2004; Nishida, 2005). In addition, ascidians have some unique features of tissue specification, such as cellulose production by the adult ectoderm, that are not found in vertebrates. These unique characteristics of ascidian development are thoroughly reviewed by Passamaneck and Di Gregorio (2005).

Specific, well-characterized genetic pathways are activated in embryos during embryonic development, and those genetic pathways are shared among the deuterostomes (Davidson and Erwin, 2006; Swalla, 2006). Examination of the timing and spatial expression of homologous genes during development can be informative in the understanding of which morphologic structures are homologous in animals with very different body plans (Gerhart et al., 2005; Rychel et al., 2006; Swalla, 2006). In the following sections, the expression of homologous genes in different deuterostome groups is discussed in the context of what these results tell us about the evolution of the vertebrates.

I. *HOX* GENE CLUSTER ORGANIZATION AND EXPRESSION IN DEUTEROSTOMES: ANTERIOR–POSTERIOR AXIS DEVELOPMENT

The *Hox* gene complex has shed light on both deuterostome relationships and the anterior–posterior homologies between body plans of the different phyla (Swalla, 2006). The *Hox* gene complex is a group of genes that are arranged from 3′ to 5′ colinearly on the chromosome and that are also expressed from anterior to posterior during embryonic development (see Chapter 9). Invertebrate deuterostomes have a single *Hox* cluster, whereas vertebrates have multiple copies (Swalla, 2006, for review). The sea urchin *Hox* cluster has been mapped, and it has undergone an inversion so that the most posterior gene, *Hox* 11/13c, is next to *Hox* 3 (Cameron et al., 2006). Hemichordates and sea urchins share motifs in their three posterior *Hox* genes, called *Hox* 11/13a, *Hox* 11/13b, and *Hox* 11/13c, which suggests that the ancestors of these two phyla had posterior gene duplications independent of the chordate lineage (Peterson, 2004; Cameron et al., 2006; Aronowicz and Lowe, 2006).

Hox developmental gene expression provides evidence for anterior–posterior homologies between echinoderms, hemichordates, vertebrates, and lancelets (Lowe et al., 2003; Aronowicz and Lowe, 2006; Swalla, 2006). Developing hemichordates express their *Hox* genes in a colinear fashion from anterior to posterior, but, instead of expression only in the dorsal neural tube, expression is seen in the entire ectoderm of hemichordates (Lowe et al., 2003). These expression patterns have been interpreted as the hemichordate ectoderm having neural potential throughout the ectoderm, such as is seen in insects (Lowe et al., 2003). In echinoderms, colinear expression has been reported in the developing adult somatopleura (Cameron et al., 2006) and in the adult nerve ring (Morris and Byrne, 2005). By contrast, tunicates show widely differing expression patterns of *Hox* genes that depend on whether the gene is expressed during the larval or adult stage (Spagnuolo et al., 2003; Passamaneck and Di Gregorio, 2005; Swalla, 2006).

II. PHARYNGEAL GILLS AND CARTILAGE DEVELOPMENT

A. *Pax 1/9* Expression and *Hox* Expression in Deuterostome Gill Slits

Pharyngeal gill slits in hemichordates were originally used as a morphologic character uniting the hemichordate enteropneust worms with chordates (Figures 6.2 and 6.4; Romer, 1967; Schaeffer, 1987; Rychel et al., 2006).

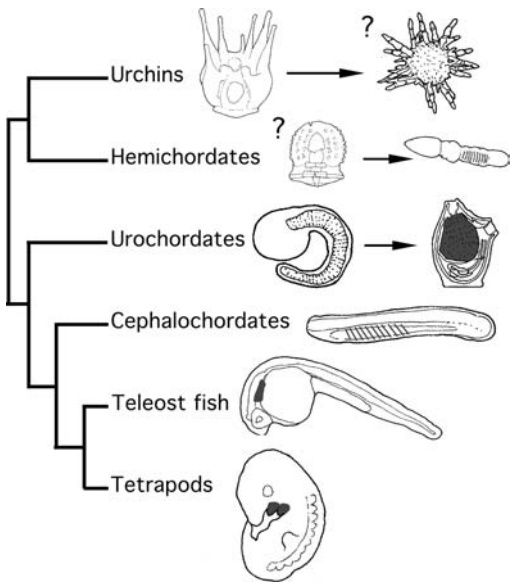

FIGURE 6.4 **The expression of *Pax1/9* in the deuterostomes.** Deuterostome phylogenetic relationships are shown from morphologic and molecular data summarized by Zeng and Swalla (2005). *Pax 1* and *Pax 9* are expressed in the developing and adult pharyngeal gill slits of all of the vertebrate gnathostomes (tetrapods and teleost fish). *Pax 1* and *Pax 9* expression patterns are shown in blue. *Pax 1/9*, a single gene that duplicated in vertebrates, is expressed in the lancelets (cephalochordates), tunicates (urochordates), and hemichordates. *Pax 1/9* has been reported to be present in sea urchins, but no expression has yet been published, so there is a question mark. Expression has not yet been reported in hemichordate tornaria larvae, so there is a question mark there as well. (See color insert.)

Structures are considered to be homologous if they have similar morphology, similar function, and similar developmental ontogenies (Rychel et al., 2006; Rychel and Swalla, 2007). The clear homology of pharyngeal gill slit structures is what causes the hemichordates to fall between the echinoderms and chordates by morphologic analysis (Figure 6.2, A; Schaeffer, 1987). The pharyngeal clefts and surrounding collagen skeleton of hemichordates, cephalochordates, and vertebrates are remarkably similar in form and function (Schaeffer, 1987; Rychel et al., 2006), but tunicates lack any cartilage skeleton in their pharyngeal structures (Figure 6.2). Developmental genetics allow for the comparison of morphologic structures at a new level: the level of genetic pathways expressed during the development of the structure. Recent work has shown that the pharyngeal slits in vertebrates, lancelets, and tunicates are all elaborated after the expression of specific *Pax* genes. The single gene called *Pax 1/9* in invertebrate deuterostomes has been duplicated in vertebrates into two genes: *Pax 1* and *Pax 9* (Neubüser et al., 1995; Holland and Holland, 1995; Ogasawara et al., 1999; Ogasawara et al., 2000a).

The expression of the paired box transcription factors *Pax 1* and *Pax 9* has been shown in the endodermal pharyngeal pouches during vertebrate development (Figure 6.4; Neubüser et al., 1995; Wallin et al., 1996; Peters et al., 1998; Ogasawara et al., 2000a). Furthermore, these transcription factors are necessary for the proper development of the pharyngeal pouches and the surrounding endodermal derivatives (e.g., the thymus) as seen by their absence in mice lacking either *Pax 1* or *Pax 9* (Wallin et al., 1996; Peters et al., 1998). There is a single homologue of these two vertebrate pharyngeal genes in lancelets (Holland and Holland, 1995), ascidians (Ogasawara et al., 1999), and hemichordates (Ogasawara et al., 1999), called *Pax 1/9*, which duplicated into the two separate copies (*Pax 1* and *Pax 9*) in vertebrates (Ogasawara et al., 1999). In both chordates and hemichordates, *Pax 1/9* is expressed in the endoderm of the pharynx and later in the pharyngeal slits (Figure 6.4). Notably, in ascidians, no expression was detected during embryogenesis. The first sign of *Pax 1/9* expression was in swimming tadpole larvae that were about to begin metamorphosis (Figure 6.4; Ogasawara et al., 1999). Likewise, expression in hemichordate adults was found to be highest in the gill endoderm (Figure 6.4; Ogasawara et al., 1999). These results suggest that the morphologic and functional similarity between the pharyngeal gill slits in hemichordates (Ogasawara et al., 1999), ascidians (Ogasawara et al., 1999), cephalochordates (Holland and Holland, 1995), and vertebrates (Neubüser et al., 1995; Wallin et al., 1996; Peters et al., 1998; Ogasawara et al., 2000b) is a reflection of similar genetic programs activated in the pharyngeal endoderm at the time of differentiation by the *Pax 1/9* or *Pax 1* and *Pax 9* transcription factors. In the light of these results and the deuterostome phylogeny (Figure 6.1), the most parsimonious hypothesis is that the deuterostome ancestor had endodermally derived gill slits and that these were subsequently lost in the echinoderm lineage (Figure 6.3, C). The mitrate carpoids, fossils from the Devonian era, do appear to have both stereoms (similar to extant echinoderms) and gill slits (Jefferies, 1986; Gee, 1996, Smith et al., 2004). Therefore, early echinoderms may have had pharyngeal gills and then lost them (Smith et al., 2004; Rychel et al., 2006). Further examination of Cambrian echinoderms for evidence of pharyngeal gills will be informative, as will the cloning and characterization of the expression of echinoderm *Pax 1/9*. No expression data have been reported in echinoderms to date

(question mark in Figure 6.4), but it will be interesting to see if this gene has been co-opted for other functions in echinoderms.

The pharyngeal gill slits share conserved transcription factors for their development as described previously and are localized in a similar manner along the anterior–posterior axis (Aronowicz and Lowe, 2006). For example, in vertebrates, *Hox 1* is first expressed at the level between the first and second pharyngeal pouch (Lowe et al., 2003). When *Hox* gene expression was examined in hemichordates, *Hox 1* was expressed between the first and second pharyngeal pouch, thereby suggesting that the location of the pharyngeal gills along the anterior–posterior axis is homologous between the hemichordates and the vertebrates (Lowe et al., 2003; Aronowicz and Lowe, 2006).

B. Pharyngeal Gill Cartilage in Hemichordates and Lancelets is Acellular

Therefore, the pharyngeal gill slits themselves appear to be homologous, but what about the cartilaginous gill bars that lie between the gill openings? The morphology and development of the gill bars in hemichordates is similar to lancelets (Schaeffer, 1987; Ruppert, 2005; Rychel et al., 2006). The bars appear as a thickening of the basal lamina between the pharyngeal endoderm, as was first reported by Hyman in 1959 and recently demonstrated by *in situ* hybridization (Rychel and Swalla, 2007). The cartilaginous bars of hemichordates stain with Alcian blue (Smith et al., 2003), but they are acellular (Rychel et al., 2006), whereas the gill bars of lamprey are made by neural crest cells and are cellular (Zhang et al., 2006). The development of gill bars in hemichordates and lancelets has been examined and it appears that their acellular cartilages are secreted by endoderm (Rychel and Swalla, 2007). This may have been the ancestral way of making cartilage in deuterostomes. Later in evolution, neural crest cells in vertebrates may have migrated into those areas and replaced the acellular cartilage with cellular cartilage. Therefore, the gill bar cartilage in hemichordates and lancelets appears to be homologous, but it is not clear whether these are homologous to any extant vertebrate cartilages (Rychel and Swalla, 2007).

III. THE POSTANAL TAIL AND THE ENDOSTYLE OF HEMICHORDATES: GENE EXPRESSION STUDIES

It is not clear how significant the postanal tail is as a defining chordate feature. Ascidians do not have an open gut as larvae, so they do not have an anus, but both lancelets and vertebrates have a postanal tail (Gerhart et al., 2005). The vertebrate and lancelet posterior *Hox* genes are expressed in the tissues of the postanal tail. Phylogenetic analyses of hemichordate enteropneust worms show that they fall into two separate monophyletic groups: those that have feeding larvae similar to echinoderms and those that are direct developers (Cameron et al., 2000). The direct-developing saccoglossids have a postanal tail and express the posterior *Hox* genes (Lowe et al., 2003), whereas the ptychoderids lack a postanal tail (Swalla, 2006). Instead, ptychoderid worms form an anus at the vegetal pole of the larvae that becomes the anus of the adult (Urata and Yamaguchi, 2004; Swalla, 2006). These results could be interpreted as evidence that the vertebrates evolved from a direct-developing hemichordate ancestor, because they are the only group of hemichordates that

show a postanal tail. However, because the hemichordates would have diverged from a chordate ancestor long before the Cambrian era (Blair and Hedges, 2005), there has been plenty of evolutionary time for the independent evolution of a postanal tail in both groups.

The endostyle present in lancelets and tunicates is thought to have homology to the vertebrate thyroid, so endostyle-specific genes have been isolated in an effort to examine this question with gene expression (Mazet, 2002; Ogasawara et al., 2000b; Sasaki et al., 2003). One of these genes is the homeobox gene thyroid transcription factor 1 *(TTF-1)*, which regulates thyroid peroxidase, the enzyme that iodinates thyroglobulin (Mazet, 2002; Ogasawara et al., 2000b; Sasaki et al., 2003). In lancelets, *TTF-1* is expressed throughout the six morphologic zones of the endostyle (Mazet, 2002), whereas, in tunicates, expression is limited to particular zones (Sasaki et al., 2003). Both the tunicate and lancelet endostyles also bind iodine, so their endostyles are considered to be homologous to the vertebrate thyroid gland (Sasaki et al., 2003; Ruppert, 2005). When the hemichordate *TTF-1* was cloned and gene expression was characterized, there was expression seen in the pharyngeal endoderm, stomochord, and hindgut (Takacs et al., 2002). The pharyngeal endoderm of hemichordates also binds iodine throughout, even in the regions that do not morphologically resemble an endostyle (Ruppert, 2005). These results could be interpreted to mean that the entire hemichordate pharynx fulfills endostyle function (Rychel et al., 2006) or that the hemichordate endostyle is not homologous to the tunicate and lancelet endostyle (Ruppert, 2005). Further developmental and functional studies will be necessary to distinguish between these two hypotheses.

IV. NO GENETIC EXPRESSION EVIDENCE FOR STOMOCHORD HOMOLOGY TO NOTOCHORD

Ultrastructural studies of the hemichordate stomochord suggested that this structure could be the homolog of the chordate notochord (Balser and Ruppert, 1990), so gene expression studies of notochord-specific genes were expected to confirm this hypothesis. *Brachyury* is a T-box transcription factor that was first isolated during mesoderm formation in vertebrates (Wilkinson et al., 1990; Holland et al., 1995) and that is expressed exclusively in the ascidian notochord (Yasuo and Satoh, 1993; Swalla, 2006). When *Brachyury T* was cloned and described in echinoderms and hemichordates, it was expressed at the site of gastrulation at the vegetal pole, which later becomes the larval anus (Peterson et al., 1999; Swalla, 2006). These results suggest that the ancestral function of *Brachyury* as a transcription factor was in promoting the gastrulation and formation of the three germ layers, and that the gene was later co-opted into notochord development (Swalla, 2006). Results from our laboratory have also shown that there is no collagen antibody staining in the stomochord, although we do see staining in the adult gill bars (Rychel et al., 2006). Unfortunately, these are all negative results, which collectively are evidence that the stomochord is not a notochord homolog; however, they do not conclusively prove it. Candidate gene expression studies so far do not suggest any other hemichordate structure as a candidate for the notochord homolog (Gerhart et al., 2005).

V. EVOLUTION OF PLACODES AND NEURAL CREST IN CHORDATES

Neural crest has been widely touted as a vertebrate innovation that allowed for the development of complicated sensory structures in the anterior head and of the skull and pharyngeal bars (Gans and Northcutt, 1983; see Chapter 26). Therefore, it has long been assumed that tunicates and lancelets would lack placodes. However, there is recent evidence from gene expression studies that tunicates have well-developed sensory placodes and lateral placodes (Manni et al., 2004; Bassham and Postlewait, 2005; Mazet et al., 2005). The buccal cavity and palps at the anterior of tunicates express *Six 1/2, Six 3/6, Eya,* and *Pitx,* which suggests a homology to the hypophyseal and olfactory placodes of vertebrates (Manni et al., 2004; Bassham and Postlewait, 2005; Mazet et al., 2005). These results suggest that the common ancestors of vertebrates and tunicates had placodes and that their anterior ends had homologous structures. A rather startling result is that the excurrent buccal opening in tunicates early on expresses *Six 1/2, Six 4/5, Eya,* and *Fox 1,* which are vertebrate markers for otic placodes, lateral lines, and epibranchial placodes (Manni et al., 2004; Bassham and Postlewait, 2005; Mazet et al., 2005; see Chapter 27). As mentioned previously, tunicate larvae do not have an open gut, so they do not have an anus during larval life. After metamorphosis, the gut is emptied out of the excurrent buccal siphon (after the tail has retracted), and the mouth forms at the anterior of the larvae. This would suggest that the adult tunicate is defecating out of its ear, which is an odd symmetry twist for a chordate.

VI. CONCLUSIONS

In summary, developmental genomics and genetics have allowed new insights into the question of chordate origins (Cameron et al., 2000; Gerhart et al., 2005; Rychel et al., 2006; Rychel and Swalla, 2007). Genomics and gene-expression studies have been extremely informative in the understanding of the homology of various structures in invertebrate deuterostomes to vertebrates. Developmental gene expression data allow one to analyze the genetic pathways that are deployed to make similar structures in genetically different organisms. Gene expression data suggest that the anterior–posterior axes of hemichordates, lancelets, and vertebrates are very similar, except that the neural genes are expressed throughout the ectoderm of hemichordates (Lowe et al., 2003). Tunicates have lost some of the middle *Hox* genes, and they express some of their *Hox* genes as larvae and some as adults; only a few are expressed colinearly (Spagnuolo et al., 2003; Passamaneck and Di Gregorio, 2005). The gill slits of hemichordates, lancelets, and vertebrates are homologous, while the gill bars of lancelets and hemichordates are both acellular (Rychel et al., 2006) and secreted by the endoderm, suggesting they are homologous (Rychel and Swalla, 2007). In contrast, tunicates completely lack gill bars in their pharyngeal region. Tunicates have been shown to have both neural and nonneural placodes, which were thought to exist only in the vertebrates. It will be interesting to examine hemichordates for the existence of placodes by examining the gene expression of homologous genes. Although tunicates are clearly chordates, they have evolved some amazing

changes in body plan, and they are likely to have lost some structures evolutionarily at the time that the tunic evolved. Hemichordates have an anterior–posterior axis similar to that of chordates, but they lack a dorsal central nervous system. Our view of the chordate ancestor is that it was a benthic worm with gill slits and a mouth that was able to filter feed but also to ingest large particles. Further research on developmental gene expression in lancelets, tunicates, and hemichordates is likely to be fruitful for the better understanding of the evolution of vertebrates.

SUMMARY

- Hemichordates are a sister group to echinoderms but not to chordates. The relationship of tunicates to vertebrates is not yet clear.
- *Hox* genes are expressed in an anterior to posterior manner in hemichordates and chordates. Tunicates have lost the middle *Hox* genes and show rather different tissue-expression patterns. Echinoderms have a rearranged *Hox* cluster and show some colinearity of expression.
- Pharyngeal gill slits in hemichordates and chordates are homologous. Pharyngeal gill bars are similar in hemichordates and lancelets, but they differ from those of vertebrates in that they are acellular.
- On the basis of gene-expression studies, the postanal tail and endostyle in hemichordates and chordates are likely to be homologous.
- Chordates specify neural and nonneural ectoderm, whereas all ectoderm is neural in hemichordates. Possible chordate notochord and neural tube homologs in hemichordates have not yet been unambiguously identified.
- Tunicates contain sensory placodes, which suggests that they have some form of neural crest and that they form a secondary anus after metamorphosis, probably from the otic placode.

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GLOSSARY OF TERMS

Deuterostome
Literally means "second mouth" (*deutero:* two; *stome:* mouth). The blastopore is formed first during gastrulation, and the mouth is formed secondarily. This

mode of development applies to all deuterostomes. Echinodermata, Hemichordata, Xenoturbellida, and Chordata are considered deuterostome phyla.

Endostyle
An endodermal structure found in invertebrate chordates in the pharyngeal area. The endostyle secretes mucus to capture small particles and to increase the efficiency of filter feeding. In lancelets and tunicates, the endostyle also accumulates iodine, and it is considered homologous to the vertebrate thyroid gland.

Graptolites
These abundant fossils are believed to be colonial hemichordates or members of the hemichordate class Pterobranchia.

Hemichordates
This phylum includes enteropneust worms and colonial pterobranchs. Hemichordates are tripartite as adults, which means that they have three body regions. The most anterior is the proboscis (protostome), then the collar (mesosome) and the posterior trunk (metasome).

Lancelets
The common name for cephalochordates. These animals are frequently referred to by the taxonomically incorrect term *amphioxus*.

Notochord
The key chordate morphologic character is the notochord. The notochord forms a stiff rod that runs from anterior to posterior in chordates beneath the dorsal neural tube, and it is usually surrounded by a sheath of extracellular matrix. The gut is found just under the notochord in vertebrates and lancelets. In lancelets and appendicularians, the notochord persists in the adult, whereas in ascidians the notochord undergoes apoptosis at metamorphosis. In vertebrates, the notochord disappears as the vertebrae develop from somites.

Pharyngeal
The area of the digestive system that serves as a respiratory and feeding organ in hemichordates, tunicates, and lancelets. The vertebrate homolog is the pharynx, which develops into gills in aquatic vertebrates, but it is the area of the throat, including the thyroid gland and thymus, in amniotes (birds and mammals).

Pharyngeal gill bars
Cartilaginous elements made of extracellular matrix and located between the pharyngeal endoderm that give the pharynx of hemichordates, lancelets, and vertebrates structure. Pharyngeal gill bars are secreted from endoderm in hemichordates and lancelets, but they develop from neural crest cells in vertebrates.

Placodes
An area of an ectodermal thickening where cells can delaminate and eventually achieve a cell fate that is not epidermal. There are both neurogenic and nonneurogenic cranial placodes, which are associated with the nervous system in vertebrates. Placodes were thought to be found only in vertebrates, but they have recently been described in tunicates using both molecular markers and careful morphologic analyses.

Pterobranch
Class Pterobranchia is the group of colonial hemichordates or pterobranchs. Colonial hemichordates reproduce both sexually and asexually, and they have feeding tentacles to capture small particles for feeding. There are many fossil pterobranchs, called *graptolites,* but there are only two extant families: Rhabdopleuridae and Cephalodiscidae.

Stomochord
A projection of the endoderm that juts forward into the hemichordate proboscis, against which the hemichordate heart beats. The stomochord cells are vacuolated, and they make an extracellular sheath.

Tunicates
A monophyletic group of animals that includes ascidians, appendicularians, and thaliaceans. This group of animals is also sometimes called *urochordates,* but *tunicates* is the preferred term. There are more than 3000 species of tunicates.

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7

UNDERSTANDING HUMAN BIRTH DEFECTS THROUGH MODEL ORGANISM STUDIES

FEYZA ENGIN[1] and BRENDAN LEE[1,2]

[1]*Department of Molecular and Human Genetics; and*
[2]*Howard Hughes Medical Institute, Baylor College of Medicine, Houston, TX*

INTRODUCTION

Birth defects are a leading cause of infant deaths. Genetic and environmental factors play an important role in the development of congenital malformations. These malformations can result from structural or metabolic–endocrine defects or from the exposure of the developing embryo to teratogens. Model organisms have long been used to understand basic conserved biologic processes. The advances in molecular techniques and the availability of fully sequenced genomes have led to the rapid translation of human disease phenotypes into animal models. The study of genotype–phenotype correlations in humans has resulted in testable hypotheses in model organisms. Comparative studies have elucidated the underlying pathogenic mechanisms of human diseases and the development of therapeutic reagents. Correct model organism selection depends on the nature of the affected developmental pathways, the relative cell autonomy of the gene defect, and the importance of tissue interactions in the pathogenesis of the disease. We discuss the strengths and weaknesses of major model organisms and how they can collectively enable the identification of the genetic and biochemical pathways involved in human birth defects.

I. DEVELOPMENTAL MALFORMATIONS

Birth defects are the leading cause of infant mortality and morbidity. Genetic and environmental factors interact to play an important role in the development of congenital malformations. These malformations can

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be caused by structural defects resulting from altered patterning, growth, or remodeling during morphogenesis, or they can result from a metabolic–endocrine defect leading to altered metabolite fluxes that secondarily affect organogenesis. Examples include mitochondria disorders, organic acidemia, and lysosomal storage diseases. Finally, exposure of the developing embryos to environmental agents, such as radiation, drugs, alcohol, and nutritional deficiencies, can also lead to severe birth defects. Recent advances in molecular biology and the availability of complete genome sequences have facilitated an increasing use of model organisms to understand the molecular mechanisms and the pathogenesis of congenital defects *in vivo*. Ultimately, genetic and epigenetic mutations can affect developmental and homeostatic mechanisms in both temporal and spatial fashions. Hence, phenotypes can arise as a result of specific disruptions during development or continued effects after in utero development. This subtle but important distinction is exemplified when considering skeletal dysostosis versus dysplasia.

To date, because of the availability of a large array of genetic tools and the strength of comparative anatomy, the mouse model has predominated in the study of human genetic diseases (Barr, 2003). However, the significant evolutionary divergence between rodents and humans has posed obstacles not only to comparative mechanistic studies but also to preclinical therapeutic models. Although nonhuman primates offer the best modeling opportunities, the lack of a genetic tool kit and high costs have made them rarely used in developmental studies. Other vertebrate models (e.g., the chick) and invertebrate models (e.g., Drosophila) have been especially powerful for the study of specific programs of organogenesis (e.g., limb development and neurodevelopment, respectively).

A. Teratogen-Induced Malformations

Teratogens can cause nonheritable malformations primarily by interfering with a gene's function during embryologic development and resulting in congenital defects. Alternatively, if the teratogen is genotoxic, heritable genetic changes that cause genetic malformations may occur (Finnell et al., 2002). Here, we list examples of the common teratogen-induced malformations and the animal models used to study them.

1. Fetal Alcohol Syndrome

Heavy alcohol consumption during pregnancy causes fetal alcohol syndrome (FAS), one of the leading known causes of mental retardation in children. Affected infants show prenatal or postnatal growth retardation (or both), central nervous system damage, learning and behavioral disorders, deficits in memory and attention, hyperactivity, speech and language delays, and characteristic craniofacial abnormalities. Phenotypic variation in FAS is dependent on the dose, pattern, and timing of teratogen exposure during embryonic development. Animal models that have been used to study FAS include nonhuman primates, rodents, large animal models (pig and sheep), chicks, fish, insects, and round worms. Although lower-order animal models have been useful for elucidating basic mechanisms, higher-order animal models have been necessary to translate these basic mechanisms into networks manifesting animal behaviors.

For example, L1, a cell adhesion molecule, is critical for normal neural development, neuroprotection, and neuritogenesis. Mutations in L1 cause severe developmental abnormalities in the human nervous system. L1 has been implicated in the pathogenesis of FAS as a result of an extensive overlap of neuroanatomic features (Bearer, 2001). Analyses of L1 gene knockout mice by different research groups demonstrate that these mice are strikingly similar to humans with mutations in the L1 gene (Dahme et al., 1997; Fransen et al., 1998). In both humans and mice, there are defects in the development of the corticospinal tract and the cerebellar vermis, hydrocephalus, and impaired learning. Ethanol has been shown to inhibit L1-mediated neurite outgrowth in cerebellar granule neurons (Watanabe et al., 2004). Such inhibition may result from decreased expression, altered cell surface distribution, impaired signal transduction, and impaired interaction with the cytoskeleton and cell adhesion molecules, which suggests that one mechanism of alcohol teratogenesis involves the inhibition of the L1 signaling cascade.

2. Fetal Valproate Syndrome

Valproic acid (VPA) is a short-chained fatty acid that is widely used in humans as an anticonvulsant and as a mood stabilizer. Exposure to VPA *in utero* has been associated with a variety of major and minor malformations, including a 20-fold increase in neural tube defects, cleft lip and palate, cardiovascular abnormalities, genitourinary defects, developmental delay, endocrinologic disorders, limb defects, and autism. The exact mechanism of how VPA causes these developmental malformations is unclear. Sodium valproate produced an increase in congenital anomalies when tested in high doses in mice, rats, rabbits, and monkeys. It has been shown that teratogenic concentrations of VPA exhibited strain-dependent effects on the expression of several genes that are important for proper embryonic development, such as cell cycle, apoptosis, and growth factor genes in mice. The administration of VPA into embryonic day 8.5 and 9.5 mice results in a failure of cranial neural tube closure, spina bifida, and limb abnormalities, such as syndactyly and oligodactyly (Ehlers et al., 1992). The variable susceptibility to malformations among inbred mouse strains suggests that genetic factors influence VPA teratogenicity, but it is unclear whether parental or embryonic factors influence these differences.

Because teratogenic effects are modified by time, dose, and duration of exposure, they can present with great clinical variability. The complex effects of teratogens on morphogenesis necessitates the use of both higher- and lower-order models that can contribute to dissecting basic mechanisms and complex behaviors.

B. Gene–Teratogen Interactions

Gene expression abnormalities can also be induced by teratogens. Well-known gene–teratogen interactions have been detailed in the studies of both large- and small-animal models, and these have contributed to the identification of key developmental pathways.

1. Sonic Hedgehog and Holoprosencephaly

The effects of cyclopamine were first described in the 1950s, when investigations were prompted by complaints about births of one-eyed lambs by

Idaho sheep ranchers. Pregnant sheep had eaten wild corn lilies, which were high in cyclopamine. Studies in mice ultimately demonstrated that this chemical interferes with the hedgehog response pathway, which, in embryos, directs the patterning of multiple organs, including the eyes and limbs.

Sonic hedgehog (Shh) is a secreted protein that has been implicated in several embryonic developmental processes. It is expressed in the floor plate of the neural tube, and it plays an important role in the patterning of the head. The evolutionarily conserved Shh pathway begins with the autoprocessing of Shh, which causes the covalent attachment of cholesterol onto the carboxy-terminus of its N-terminal domain. After proteolytic cleavage, the active Shh ligand binds to the Ptch receptor, and, subsequently, Gli transcription factors are activated. Aberrations in the Shh pathway in humans during embryogenesis can cause a severe congenital malformation of the forebrain known as holoprosencephaly (HPE; Roessler et al., 1996). Family members carrying identical mutations in *SHH* can exhibit a different malformation spectrum of HPE, with some being minimally affected and other being severely affected, such as those with cyclopia. This intrafamilial variability in phenotype may result from modification by genetic background or environmental factors.

Defects of cholesterol synthesis also result in HPE in Smith–Lemli–Opitz syndrome, which results from an inherited defect in 7-dehydrocholesterol reductase, an enzyme that catalyzes the last step of cholesterol synthesis (Fitzky et al., 1998; Waterham et al., 1998). Moreover, low maternal cholesterol levels and the gestational use of the cholesterol-lowering statin drugs was associated with HPE (Edison and Muenke, 2004a; 2004b). However, it has been shown that these teratogens did not prevent the sterol modification of Shh during autoprocessing but rather inhibited the response of target tissues to *Shh* (Cooper et al., 1998). Here, observations of teratogenic effects in a large-animal model help to identify a key ubiquitous signaling pathway during development.

2. Steroid/Thyroid/Retinoid Superfamily of Transcription Factors

Another classic teratogen that has identified important pathways is retinoic acid. Retinoic acid is a very potent teratogen, and it has itself been implicated as an endogenous developmental signaling molecule in vertebrate embryos. The consequences of exposure to retinoids during human pregnancy were seen during the early 1980s, when the drug Accutane (isotretinoin or 13-*cis*-retinoic acid) was used for the treatment of cystic acne. Women who took that drug had a number of spontaneous abortions, and affected infants were born with a variety of birth defects, including craniofacial, cardiovascular, and central nervous system defects, which were referred to as *retinoic acid embryopathy.* Either an excess or a deficiency of retinoids has been shown to cause abnormal morphologic development (Lammer et al., 1985). The mechanism of action of retinoic acid–induced teratogenesis has yet to be fully elucidated. It is known that retinoic acids exert their pleiotropic effects by binding to two families of nuclear receptors: the retinoic acid receptors (RARs) and the retinoid X receptors. Each receptor type has a number of isoforms that are produced by alternative splicing and differential promoter usage. It is likely that the improper activation of RARs might cause retinoid acid–induced teratogenesis by affecting different developmental processes such as cell proliferation, differentiation, and apoptosis. Here, mouse

knockout models have been powerful for the elucidation of components of this pathway. The knockout of multiple RARs in mice recapitulated defects that were characteristic of vitamin A deficiency in human embryos.

C. Environmental Factors and Genetic Susceptibility

Gene–environment interactions play an important role in congenital malformations. Neural tube defects (NTDs) and craniofacial anomalies are good examples of such interactions. Numerous studies have shown that the risk of having a child with NTD can be significantly reduced by folic acid supplementation during pregnancy. However, the specific molecular mechanisms that lead to the protective effects of folic acid are unknown. Several genetic polymorphisms have been found to be associated with defects in folic acid–dependent homocysteine metabolism. The methylenetetrahydrofolate reductase 677C>T and 1298A>C polymorphisms result in elevated endogenous homocysteine levels, and they are associated with an increased risk of NTD. Environmental factors also play a role in the etiology of craniofacial defects, such as cleft palate and cleft lip. Orofacial clefts have been associated with maternal cigarette smoking, alcohol consumption, and a lack of folic acid supplementation. Human maternal periconceptional intake of multivitamins containing folic acid has been associated with a reduction in the risk of delivering infants with clefts; however, this reduction in risk has not been observed in all studies. The transforming growth factor alpha (TGFα) genotype has also been shown to be important for contributing to the development of cleft palate. Some genetic studies have shown a two- to five-fold increased risk of clefting among individuals with the less-common allele for TGFα. TGFα is a secretory protein that binds to the epidermal growth factor receptor, and it has been localized to palatal epithelium during mouse palatal closure. Studies using targeted knockout mouse models and spontaneous mutants have been powerful in the validation of the importance of TGF signaling in clefting (i.e., TGFβ3$^{-/-}$ mutant mice exhibit clefting; Proetzel et al., 1995). Moreover, the discovery of numerous mouse mutants with cleft palate has confirmed the complex genetics of this trait and the multiple signaling pathways that are involved with clefting. Although human and mouse cleft development is remarkably similar, mice do not develop cleft lip.

II. MODEL ORGANISMS

A. Primary Model Organisms in the Study of Development and Disease

1. Unicellular Organisms

a. Yeast

The budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe* are single-celled fungi with distinct life cycles. *S. cerevisiae* is the first eukaryotic organism for which the entire genome sequence was completed (Goffeau et al., 1996). The yeast genome contains about 6000 genes, and about 20% of human disease genes have counterparts in yeast. Yeast's rapid generation time and simple and inexpensive maintenance under laboratory conditions make it advantageous for both classic genetic studies and high-throughput genomic approaches. *S. cerevisiae* provides a

TABLE 7.1 Strengths and Weaknesses of Model Organisms

Species	Strengths	Weaknesses
Unicellular organisms		
Saccharomyces cerevisiae	Homologous recombination; powerful genetic and proteomic technologies; complete genome sequence; simple and inexpensive maintenance; basic eukaryotic cell organelles present; cell cycle control similar to animals	Unicellular; no distinct tissues
Dictyostelium discoideum	Powerful genetic and proteomic technologies; simple biologic processes similar to animals	Unicellular; no distinct tissues
Invertebrates		
Caenorhabditis elegans	Excellent genetics; effective RNA interference; powerful molecular techniques; complete genome sequence; suppressor/enhancer screens; fully known morphology; transparent; small number of cells and all cell lineages characterized; long-term storage (−80°C); known neuronal connectivity	No homologous recombination; difficult gene-expression analysis
Drosophilia melanogaster	Powerful genetics; molecular techniques; complete genome sequence; suppressor/enhancer screens; mosaic analysis; effective RNA interference; easily generated transgenics; well-characterized development	No embryo freezing; difficult embryologic manipulations; difficult targeted gene disruptions
Vertebrates		
Danio rerio	Vertebrate; external fertilization; large number of eggs; transparent; accessible developmental stages; organ systems similar to higher vertebrates; morpholinos; RNA interference; mutagenesis screens	No homologous recombination; difficult to generate transgenics
Xenopus laevis	Vertebrate; external embryonic development; large size; identifiable blastomeres; easy embryo manipulations	Long time to sexual maturity; no genetics; difficult to generate transgenic animals
Gallus gallus	Descriptive embryology; ideal for embryologic manipulations (e.g., transplants of limbs, neural crest, notochord)	Limited genetics
Mus musculus	Vertebrate; development, cell types, and tissues similar to human; powerful genetics; targeted gene disruption by homologous recombination; transgenic technologies; large mutant collection; source for primary cell cultures	Development is in utero; expensive maintenance; relatively long time to sexual maturity and maturity; early embryonic lethal phenotypes difficult to study (resorption *in vivo*)

significant advantage for experimentation because of its fast and easy means of gene cloning, gene disruptions, gene overexpressions, and single-step gene replacements (Table 7.1). Because it can grow as either haploids or diploids, recessive mutations can be identified by phenotypic changes in the haploid strain. In addition, complementation analyses can be performed in yeast. In contrast with mammalian cells, in which redundant processes are often obstacles to understanding the function of a specific gene, yeast provide a clean readout against a null background. As a result of the high degree of conservation of basic molecular and cellular mechanisms between yeast and human cells, it represents a highly useful system for the investigation of cell architecture and fundamental cellular mechanisms. The genes controlling the eukaryotic cell cycle were identified mainly through studies of the yeasts *S. cerevisiae* and *S. pombe*. Yeast has proven to be an extremely good model for cancer studies, because its mechanism of cell division and its response to DNA damage are similar to human cells. It is also used as a model in apoptosis, aging, and DNA repair studies. However, limitations exist, because not every human disease gene has an ortholog in yeast, and pathologies that affect specific tissues, organs, and physiologic functions cannot be assessed at the single-cell level. Moreover, as a result of its unicellularity, the functions of the genes that are expressed as different isoforms in different cell types cannot be analyzed in yeast. As a whole, yeast has been an excellent model for studying conserved biologic cell mechanisms that affect developmental processes in a cell-autonomous context. They have also proven useful for translating the consequences of human mutations that act in a non–cell-autonomous fashion at the tissue or organ level.

b. Dictyostelium discoideum

Dictyostelium discoideum amoebae thrive in moist soil. Nutritional stress drives cells to aggregate by means of chemotactic signals, and these aggregates then differentiate into multicellular fruiting bodies that contain spores. Its recently published genome encodes approximately 12,500 genes. Although this is more than twice the number of genes in yeast, it is still only about half that of humans, and the rarity of alternative splicing simplifies its proteome even further as compared with those of vertebrates (Eichinger et al., 2005). However, efficient genetic manipulations by gene targeting and replacement as well as by insertional mutagenesis, suppressor screens, and RNA interference (RNAi) make Dictyostelium a popular experimental system. Dictyostelium exemplifies many processes that are characteristic of complex eukaryotes, including cytokinesis, motility, phagocytosis, chemotaxis, signal transduction, and aspects of development such as cell sorting, pattern formation, and cell-type determination and differentiation. It has been used by researchers to study the mechanisms of action of myosin mutations that cause cardiac myopathies, the molecular basis of cisplatin (a drug used for the treatment of cancer), and the mechanism of action of lithium and VPA, which are used for the treatment of depressive disorders (Egelhoff et al., 1993; Eickholt et al., 2005; Li et al., 2000). It has also been established as a host model for the pathogenesis of infectious diseases such as malaria, Legionnaire's disease, salmonellosis, tuberculosis, listeriosis, and pseudomoniasis. Although Dictyostelium is a very good model for the study of simple cellular behaviors, its limited cellular diversity and its absence of distinct tissues limit its use as a model for eukaryotic cell function.

2. Invertebrate Models

a. Caenorhabditis elegans

Caenorhabditis elegans is a small soil nematode with a short life cycle of 3.5 days at 20°C. Adult hermaphrodites of this species can give birth to more than 300 progeny by self-fertilization. The worms are very easy to grow in the laboratory on agar medium in Petri dishes, and stocks can also be frozen, thereby allowing one to create large numbers of mutant strains with limited maintenance. Its cell lineage, the complete connectivity of its nervous system, and its nerve–muscle synaptic connections are known; this has made it a powerful model for studying basic neurodevelopmental mechanisms. As a result of its transparency, it is possible to view internal structures, especially with enhancement by green fluorescent protein and differential interference contrast in live animals. The genome sequence of *C. elegans* was completed in 1998, and it revealed that 43% of *C. elegans* genes have human orthologs, including numerous disease genes (Culetto and Sattelle, 2000). A major strength of using *C. elegans* as a model system is that genetic manipulations are easily performed and tools are well developed. The ability to breed a mutation to homozygosity in a single generation facilitates the performance of genetic screens for recessive mutations more readily than in other organisms. The powerful gene-mapping strategies based on single nucleotide polymorphism detection can also be performed in worms. Rapid cosmid rescue-transformation of mutant animals by microinjection is also available. RNAi is effectively and easily applied to *C. elegans* in the knockdown of specific genes. The organism is also well suited for second-site suppressor/enhancer screens that facilitate the determination of components of a genetic pathway after a single gene involved in that process has been identified (Jorgensen and Mango, 2002).

C. elegans has been extremely powerful in the study of the apoptotic pathway in higher eukaryotes, because the key components of the apoptosis machinery appear to be conserved between humans and nematodes. Many genetic diseases involve the dysregulation of apoptotic programs, such as sclerosis, type I diabetes mellitus, Hashimoto thyroiditis, Sjögren syndrome, and certain cancers (e.g., melanoma), thereby making *C. elegans* an important model for elucidating their pathogenesis. *C. elegans* has also been used as a model system for studying the mechanisms of aging, neurodegenerative diseases, muscular dystrophy, polycystic kidney disease, and other human diseases.

Despite its unique advantages, *C. elegans* has limitations as a model organism. One of these limitations is, of course, its relative divergence from humans as compared with that of another much-studied invertebrate, the fly. Also, difficulties in the direct analysis of gene expression and the performance of embryologic manipulations are other experimental limitations.

b. Drosophila melanogaster

The fruit fly *Drosophila melanogaster* has been used by researchers for more than 100 years in the areas of gene discovery and genetic analyses. Since the completion of the genome sequence of Drosophila in 2000, 61% of its genes have been shown to have human counterparts (Adams et al., 2000). Its rapid generation time and the availability of various forward and reverse genetics approaches make Drosophila a powerful model organism. Most of

the studies in the fly have been performed by using forward genetics, in which the chemical mutagen EMS or P-element transposition are used in large-scale phenotype-based mutagenesis screens. The availability of single nucleotide polymorphism maps has also been useful for mapping specific mutations. P-element transposons have also been used to screen for second-site modifiers (enhancers and suppressors) of a specific sensitized background, which permits the identification of new genes involved in a given developmental pathway or process. Large collections of P-element insertion stocks have been generated, thereby allowing for the direct screening for mutant phenotypes. Misexpression and overexpression phenotypes have been generated in a spatiotemporal fashion using the GAL4-UAS system. Finally, clonal analyses have been extensively used in Drosophila to trace cell lineages, to analyze lethal mutations at later stages of development, and to distinguish cell-autonomous versus nonautonomous actions of genes after applying the FLP/FRT recombination system in somatic lineages. Loss-of-function studies of a particular gene have been analyzed by different methods, such as the imprecise excision of P-elements, targeted gene replacement, or RNAi. RNAi has been shown to effectively block gene expression *in vivo* in Drosophila. It has been shown that 75% of human genes known to be associated with disease have a Drosophila ortholog (Reiter et al., 2001). Moreover, Drosophila shows similarities to humans in basic biologic cell processes, including gene expression, membrane trafficking, cytoskeleton organization, extracellular matrix, determination of cellular asymmetry, epithelial organization, neuronal connectivity, synaptic function, cell–cell and intracellular signaling pathways, and apoptosis.

Arguably, Drosophila has been the model organism of choice for dissecting the genetic pathways that affect neurodevelopment. Recently, Drosophila has been applied to identifying mechanisms of human neurodegenerative disease, including Alzheimer, Parkinson, and Huntington diseases. Neurodegenerative diseases share common features. They are caused by dominant mutations; they exhibit a late onset and progressive neuronal degeneration, and they are associated with the formation of highly stable protein aggregates. In the case of polyglutamine diseases, repeat length correlates with the severity of the phenotype. It has been shown that flies can mimic human pathology in several respects. Transgenic flies expressing polyglutamine repeats showed that increased repeat length causes neural degeneration, and degeneration is typically seen in late Drosophila development (Marsh et al., 2000). As in the human, condition neuropathology in the fly is progressive, and protein aggregates form upon the expression of mutant polyglutamine repeat peptides (Davies et al., 1997; Marsh et al., 2000).

Studies with Drosophila have shown that flies are helpful for the sharing of mutant phenotypes that are similar to those of human diseases and also for facilitating the identification of the components of a given developmental pathway. For example, when the adenomatous polyposis (APC) gene, which is responsible for numerous intestinal polyps that predispose individuals to colon cancer, was identified by positional cloning in 1991, its function was unknown. Later, the identification of APC's interaction with β-catenin provided the first clues about its function. However, the link between the Wnt signaling pathway and APC was established after β-catenin (Armadillo in Drosophila) and the Wnt signaling components were discovered by genetic analyses in Drosophila. It is now known that the Wnt pathway plays a critical role in the pathogenesis of colon cancer. Similarly, the identification of the

Notch, Shh, and Nodal pathways in Drosophila has shed light on many aspects of the dysregulation of these signaling mechanisms in human biology and disease.

Although Drosophila has been a very powerful model organism, several limitations exist. Flies differ from humans by their much simpler circulatory systems, immune responses, skeletal systems, and cognitive abilities. Numerous subtle differences exist in the functions of individual proteins. Humans often have several copies of a gene that is present in only one copy in the fly genome. The different forms of many human genes have evolved to acquire different expression patterns and unique functions in different cell types. For example, unlike Drosophila, there are three hedgehog proteins (Shh, Indian hedgehog [Ihh], and Desert hedgehog [Dhh]) in mammals. Among these, Ihh has been shown to be important for the differentiation of prehypertrophic chondrocytes, which is a cell type that is not found in flies. In fact, skeletal development serves as a contrast with neurodevelopment when considering the relevance of the Drosophila model to human disease.

3. Vertebrate Models

a. Danio rerio (zebrafish)

Zebrafish *(Danio rerio)* has been an attractive vertebrate model system because of its simple and inexpensive maintenance at high densities in the laboratory and the large numbers of embryos that can be produced in a very short time. Zebrafish embryos develop most of the major organ systems—including the cardiovascular, nervous, and digestive systems—in less than a week. The transparent embryos develop externally, which allows for direct observation of the morphologic defects. Although targeted gene knockout technology has not yet been developed for zebrafish, specific genes can be knocked down transiently by the injection of morpholino antisense oligomers. High-resolution simple sequence length polymorphisms and radiation hybrid maps are available to help with the genetic mapping of mutations. Large-scale forward genetic screens have been generated in zebrafish by using N-ethyl-N-nitrosourea (ENU) and insertional mutagenesis. The mutants that were obtained from these screens showed embryonic patterning and organ system defects of the retina, bone, cartilage, brain, hematopoietic system, digestive system, and cardiovascular system. These mutants represent a useful tool for identifying genes that are involved in human disorders. Zebrafish embryos have long been a model for studying teratogen-induced malformations such as human FAS. The characteristic features of human FAS, such as brain defects, are also observed in zebrafish that are exposed to ethanol. It is a useful model not only for dissecting disease-associated genes and pathways but also for testing for environmental toxins in humans, for drug-target identification, and for the *in vivo* validation of targets before clinical trials (Blader and Strahle, 1998).

b. Xenopus laevis and chick

Xenopus laevis is a nonmammalian vertebrate that has been used as a model system as a result of its several advantages, including external embryonic development, large size, and easy experimental manipulations. It has been used for the study of embryonic development, the patterning of the basic body plan, the determination of cell fate, and the early patterning of major

organs (e.g., the digestive, circulatory, and nervous systems). Studies in Xenopus have helped elucidate the formation and function of Spemann's organizer. Similar to zebrafish, loss of function can be generated by morpholino oligos, but gene targeting is not yet widely available.

Another nonmammalian vertebrate model is the chick. The chicken has significant advantages as a model because of its low cost and availability, its fully sequenced genome, and its simple physical manipulations *in ovo*. Direct access to the embryo facilitates the removal of tissue, the implantation of morphogen-soaked beads, heterotopic transplantations, and both viral and nonviral mediated gene transfer to tissues. The chicken has proven to be a superb model organism for the study of limb development, and it has enabled the identification of key organizing centers, such as the zone of polarizing activity and the apical ectodermal ridge. It has also been used in the study of viruses and cancer. The first tumor virus (Rous sarcoma virus) and oncogene *(src)* were identified in the chicken. However, one of the disadvantages of these nonmammalian vertebrate animal models is the relative divergence of some genes and their functions from that seen in humans. Moreover, direct genetic manipulations are difficult to perform, which can be a problem during the creation of transgenic strains and targeted gene disruptions.

c. Mouse

The completion of the mouse genome sequence in 2002 demonstrated that 80% of mouse genes had a single human ortholog (Waterston et al., 2002). Well-developed genetic manipulations and the ability to use powerful molecular tools made the mouse an even more valuable model system (Bedell et al., 1997). ENU-induced point mutations have been successfully used to generate large-scale chemical mutagenesis screens (Justice et al., 1999). These screens enabled the production of allelic series of mutations that complemented classical loss-of-function alleles with hypomorphic and neomorphic alleles. Thus, the potential embryonic lethality of recessive null mutations could be circumvented. *In vitro* manipulations of embryonic stem cells (ESCs) allowed for the application of reverse genetic approaches, including of knockout technology and insertional gene trapping. Homologous recombination-based gene targeting had the greatest initial impact on the understanding of gene function in mice. Although loss-of-function studies have provided important information about many human diseases, they have been less informative in situations involving genetic redundancies and early embryonic lethality. The lethality of recessive null alleles prevented the analysis of the later functions of a gene. However, this has been ultimately circumvented by generating conditional alleles by incorporating Cre recombinase-mediated Lox P excision in the targeting vector or by isolating point mutations from ENU screens.

Abundant comparative genetic studies between human and mouse mutant alleles have revealed cases of both excellent and poor phenocopies. In some cases, both the inheritance pattern and the phenotypes correlated well. In others, phenotypic differences and incomplete penetrance could be observed. For example, variable phenotypicexpressivity in addition to strain-dependent penetrance can often be observed in mouse models. Factors that affect the degree of phenotypic correlation between mice and humans include the following: 1) differences in the dosage sensitivity of the mutation and the affected pathway; 2) differences in redundant pathways and genes; 3) genetic and

epigenetic modifiers specific for inbred strains of mice versus outbred humans; 4) true divergent function of the target gene in mouse versus humans; and 5) differential effects seen early versus later in development.

Although knockout mice might present with a more severe phenotype than the human phenotype or have no clinical phenotype at all, differences may be used advantageously for identifying modifiers, alternative developmental pathways, and novel gene interactions. An example is the Lesch–Nyhan syndrome mouse model. Lesch–Nyhan syndrome is an X-linked disorder characterized by hyperuricemia, choreoathetosis, spasticity, and mental retardation that results from a complete lack of hypoxanthine–guanine phosphoribosyltransferase (HPRT). HPRT knockout mice are phenotypically normal and healthy, with only subtle changes in brain dopamine levels. However, they provided a good biochemical model for studying the *in vivo* consequences of HPRT mutation on metabolite alterations (Jinnah et al., 1994).

In contrast with the generation of loss-of-function mutations, gain-of-function mutants have been primarily generated in a targeted fashion by pronuclear injection into generate transgenic mice. Here, mice have served as valuable models of overexpression of wild-type and/or mutant proteins. The use of tissue-specific promoters to direct transgene expression has enabled the misexpression of proteins in tissues not normally expressing the target gene, whereas the development of BAC transgenesis has enabled more physiologic overexpression models.

Despite its many advantages as a model system and its similarity to humans in both anatomy and physiology, the mouse is far from the perfect experimental system. Unlike lower-order vertebrates, early organogenesis, differentiation, and development are hard to observe, because these processes take place *in utero*. Dissecting early essential functions during embryogenesis from later tissue-specific or organ-specific functions can be challenging and may require lengthy genetic manipulations and breeding schemes. Finally, mice have a comparatively longer life cycle, and colonies are relatively expensive to maintain in laboratories. As an alternative rodent model, the laboratory rat *(Rattus norvegicus)* has been a long-established model for studying human disease and physiology. Unfortunately, gene-targeting studies in the rat have been limited by the lack of availability of embryonic stem cells and technical inefficiencies. However, transgenesis has been achieved by microinjection and, more recently, by retroviral/lentiviral integration.

The ability to move mouse/rodent modeling of human disease into an accelerated phase will be facilitated by the widespread availability of null mutations for all genes and then by the generation of a series of allelic mutations in each gene. To achieve this latter goal, the ability to rapidly generate targeted single nucleotide substitutions in ESCs will be important. Progress in new technologies, such as *in vivo* RNAi using lentiviral transfer into ESCs and pronuclei, is providing novel avenues for generating hypomorphic and loss-of-function alleles more rapidly. However, off-target effects as well as potential unwanted effects on the endogenous microRNA processing machinery have yet to be evaluated.

B. Mouse Models for Human Birth Defects

Developmental defects arising from genetic mutations can be broadly divided into those with structural origins and those with metabolic origins.

1. Structural Defects

Structural defects are congenital malformations that result from altered patterning, differentiation, proliferation, remodeling, and the apoptosis of affected tissues during development. Resulting phenotypes can be associated with most cellular processes, including the following: 1) morphogen and morphogen antagonists; 2) morphogen–receptor interactions; 3) signal transduction downstream of receptor–ligand interactions; 4) transcription; 5) RNA processing, posttranslational modification, and trafficking; and 6) matrix production and cell matrix interactions. Skeletal malformations exemplify a common class of structural birth defects, and developmental dysplasias of the skeleton can be used to illustrate the salient features that are characteristic of this class.

a. Skeletal dysplasias as a model for comparative mouse–human genetic analyses

Formation of the skeleton requires the differentiation of the mesenchymal stem cell via either the chondrogenic lineage or the osteoblastic lineage. Chondrogenesis involves differentiation first into chondroblasts, then into proliferating chondrocytes, next into prehypertrophic chondrocytes, and finally into hypertrophic chondrocytes during the process of endochondral ossification (see Chapter 39). Terminally differentiated hypertrophic chondrocytes undergo apoptosis and are replaced by mineralizing bone. By contrast, intramembranous ossification involves the direct differentiation of the stem cell into preosteoblasts, early osteoblasts, mature osteoblasts, and, finally, into terminally differentiated osteocytes. Both cell differentiation processes must be patterned in the body plan's three-dimensional space (i.e., proximal–distal, anterior–posterior, and dorsal–ventral).

Two critical transcription factors directing the specification of the mesenchymal stem cell to the respective chondrogenic or osteoblastic lineages are *Sox9* and *Runx2*. In humans, haploinsufficiency of *SOX9* (a transcription factor with an sex determining region Y [SRY]-related high-mobility group [HMG] box domain) causes campomelic dysplasia. Campomelic dysplasia is an autosomal dominant neonatal chondrodysplasia characterized by a severe dwarfism that affects all cartilage-derived structures and that is also characterized by frequent male-to-female sex reversal. During mouse embryonic development, *Sox9* is expressed in all prechondrocytic mesenchymal condensations. Later, its expression is maintained at high levels in fully differentiated chondrocytes. $Sox9^{+/-}$ mice have been generated by classic gene-targeting strategies. Heterozygous *Sox9* mice die perinatally and phenocopy most of the skeletal abnormalities seen among patients with campomelic dysplasia, including cleft palate, hypoplasia, and the bending of many skeletal structures (Bi et al., 2001). Moreover, studies using *Sox9* knockout chimeric mice showed that mesenchymal cells that did not express *Sox9* were unable to differentiate into chondrocytes or to contribute to mesenchymal condensations, which suggests that *Sox9* is essential for chondrocyte differentiation and cartilage formation (Bi et al., 1999).

Runx2 is a runt domain transcription factor that is essential for osteoblast cell fate commitment and chondrocyte maturation. It is expressed in osteochondro progenitors, developing osteoblasts, and in a subset of chondrocytes. The haploinsufficiency of *RUNX2* in humans causes dominantly inherited cleidocranial dysplasia (CCD). CCD is characterized by skeletal anomalies (including open fontanels), late closure of cranial sutures with Wormian bones, delayed eruption of permanent dentition, rudimentary clavicles, and

short stature. Several mouse models of *Runx2* function have been generated. *Runx2* null mice show a complete lack of bone formation as a result of a maturational arrest of osteoblasts (Komori et al., 1997; Otto et al., 1997). These mice have only cartilage anlagen of the skeleton and no evidence of osteoblasts or mineralization. Heterozygous mice phenocopy CCD with delayed ossification of the fontanels and hypoplastic clavicles. In addition, transgenic mouse models demonstrated that *Runx2* is also important for postnatal bone development. The overexpression of *Runx2* in osteoblasts caused osteopenia and fractures in transgenic mice (Liu et al., 2001), whereas it has been shown that the overexpression of *Runx2* in cartilage induced chondrocyte hypertrophy while partially rescuing the chondrocyte maturation defects in *Runx2* null mice (Takeda et al., 2001). These data demonstrate a complex spatiotemporal function of *Runx2* in skeletal development. The function of *Runx2* in chondrocyte maturation has been confirmed in humans. Both decreased hypertrophy and decreased expression of *RUNX2* target genes were found in human CCD cartilage (Zheng et al., 2005). In general, there is an excellent correlation between mouse and human mutations for these two transcription factors. This reflects the strict dosage requirement for many transcription factors in general and the often near-complete penetrance of phenotypes associated with alterations in transcription-factor expression levels. In fact, most human transcription-factor diseases are dominantly inherited, and the majority are the result of loss-of-function mutations. Similarly, most corresponding mouse mutants are semidominant and faithfully phenocopy the human condition.

The pathogenic consequences of transcription factor aberrations ultimately reflect the dysregulation of their target transcriptional network of genes. Considering skeletogenesis, key targets for *Sox9* and *Runx2* include matrix structural proteins. It is not surprising that mutations in the matrix structural proteins were the first identified in the osteochondrodysplasias, but, because of the complexity of matrix-cell and matrix-environment interactions, comparative mouse–human studies have been insufficiently robust to reveal all influences. For example, the tetraped versus the biped nature of rodents versus primates dictates different biomechanical forces impinging on the phenotypic expression of matrix alterations in the skeleton. In fact, the tremendous clinical variability associated with both fibrillar collagen mutations in humans points to the contribution of many modifying factors. Human mutations of type I collagen in the *COL1A1* and *COL1A2* genes cause osteogenesis imperfecta (brittle bone disease). Type II collagen *(COL2A1)* mutations cause disproportionate dwarfism or chondrodysplasias of varying severities (achondrogenesis type II, hypochondrogenesis, spondyloepiphyseal dysplasia congenita, Kniest dysplasia, and Stickler syndrome spectrum). Still, observations from mouse studies have affected our understanding of the underlying disease mechanisms. For example, subtle substitution mutations in the fibrillar collagens act in a dominant negative fashion, producing a severe phenotype. Large deletions and null mutations cause a quantitative loss-of-function effect and are associated with milder phenotypes replicated in the first *COL1A1* mouse models. Retroviral insertional mutants that affected levels of *COL1A1* expression caused mild osteogenesis imperfecta in mice, whereas transgenic mice harboring additional copies of the gene with engineered point mutations had severe osteogenesis imperfecta (Bonadio et al., 1990; Stacey et al., 1988). As "knock-in" technologies have improved, the replacement of the wild-type allele with a point mutation

replicating human alleles has become a powerful approach for modeling. However, there are still significant limitations for mouse models in the study of human disease. Of note, type X collagen *(COL10A1)* mutations cause Schmid metaphyseal chondrodysplasia via dominant loss-of-function mechanisms. Interestingly, mice that are null for *COL1A1* appear to be phenotypically normal (Rosati et al., 1994).

Morphogen signaling occurs upstream of transcriptional networks. A primary signaling pathway regulating chondrocyte proliferation and differentiation is the fibroblast growth factor signaling pathway. Recurrent *FGFR3* mutations have been shown to cause most cases of achondroplasia, which is the most common form of disproportionate dwarfism. Ultimately, the mouse models for *Fgfr3* mutations elucidated the pathogenesis of this disorder. Fibroblast growth factor signaling negatively regulates chondrocyte proliferation, and the achondroplasia mutation is an activating mutation. A complete loss of function of *Fgfr3* in mice caused overgrowth of the growth plate (Colvin et al., 1996). By contrast, transgenic mice expressing the achondroplasia mutation as well as knock-in mutants phenocopied human achondroplasia (Naski et al., 1996; Wang et al., 1996).

2. Metabolic–Endocrine Defects

Developmental defects can also be associated with classic inborn errors of metabolism. These diseases are characterized by deficiencies of enzymes and transporters that result in the dysregulation of metabolite flux. The consequent accumulation of toxic upstream precursors, the deficiency of downstream products, and/or the stimulation of alternative metabolic pathways occur and exceed the threshold for clinical disease. Major categories of disease include organic acidemias, peroxisomal disorders, lysosomal storage disorders, carbohydrate metabolism disorders, amino acidopathies, fatty acid oxidation defects, mitochondrial/respiratory chain defects, and urea cycle disorders (Lanpher et al., 2006). Some conditions can cause structural developmental defects, especially in target organs like the liver, muscle, and brain. Others may be primarily associated with metabolic decompensation and/or chronic neurologic symptoms. Modeling these diseases in the mouse has represented a considerable challenge. The divergence of metabolic pathways and the differential use of alternative disposal pathways allow for a significant discordance in phenotypes between mouse and man, as previously exemplified by the HPRT enzyme. Galactosemia and urea synthesis provide excellent examples, comparing in the one case the difficulty and the other the facility of using mouse modeling for the study of human inborn errors of metabolism.

Galactosemia is an autosomal recessive disease of carbohydrate metabolism that results from a deficiency of the enzyme galactose-1-phosphate uridyltransferase *(Galt)*. Galactose and the derived toxic products galactose-1-phosphate and galactitol accumulate in the blood, leading to neonatal morbidity and mortality. Even with tight dietary control, patients suffer long-term morbidity that is likely related to a deficiency of downstream product and dysregulation of the glycosylation machinery. In affected infants, symptoms present soon after the ingestion of a lactose-based formula or breast milk. Homozygous individuals exhibit vomiting, rapid weight loss, hepatomegaly, and jaundice. Long-term complications include mental retardation, cataracts, hepatomegaly, and ovarian failure. Animal models have been used to better

understand galactosemia. Animal studies typically involve high-galactose diets overloading the galactose metabolic pathway. Additionally, *Galt* knockout mice have been generated and studied (Leslie et al., 1996). However, *Galt* null mice appear to be normal and to have no evidence of neonatal toxicity. Even after keeping the animals on a high-galactose diet for weeks, no obvious phenotype was observed. The knockout of other enzymes in this pathway, such as galactokinase, also fails to fully phenocopy the human condition. Hence, these data suggest the evolution of alternative galactose handling pathways in mice in addition to the classic *Galt*-mediated oxidation. Interestingly, there are more examples of such discordance in metabolism.

In contrast with the galactose metabolism pathway, urea synthetic pathways have been relatively well conserved in mice and humans. Urea cycle disorders (UCDs) are caused by the deficiencies of the enzymes required for transferring nitrogen from ammonia and aspartate into urea. Initial signs of UCDs in infants include somnolence, poor feeding, vomiting, seizures, lethargy, and coma. Persistent hyperammonemia, if not treated, may cause irreversible neuronal damage. Knockout mice for most of the enzymes of the UCDs have been generated, and they faithfully replicate the neonatal hyperammonemic phenotype. However, whether they might also replicate the long-term morbidity of UCDs that is not directly attributable to hyperammonemia is unknown because of the perinatal lethality of the mutants.

Animal models of inborn errors of metabolism have proven in general to be excellent models for the development of therapy. Numerous metabolic disease models have been used for the development of protein replacement and gene therapies, and substantial long-term correction has been observed in many of them. Where they have often failed is in predicting the host toxicity to therapy. Hence, there are different therapeutic indices for a given specific therapy when moving from small-animal to large-animal models. This is because toxicity to treatment—especially to biologic therapies—cannot always be predicted reliably in rodents. Ultimately, although small-animal models such as rodents are excellent from the perspective of assessing efficacy, toxicities should be evaluated in nonhuman primate models and humans in phase I settings. Examples of successes in genetic therapies developed with the help of mouse models include protein replacement therapies for several of the lysosomal storage diseases, including the mucopolysaccharidoses. Still, there are examples in which the mouse model does not replicate human pathology, and this has been a major obstacle to the development of therapy. This is most prominently reflected in the cystic fibrosis transmembrane regulator mouse model of cystic fibrosis, in which lung pathology is absent (Snouwaert et al., 1992).

III. PERSPECTIVES

Integrating the study of human genetic disease with comparative analyses of model organisms has proven to be a powerful approach to elucidating basic developmental mechanisms, understanding the pathogenesis of disease, and testing novel therapeutic approaches. The choice of model organism needs to consider the nature of the studied pathway, whether the mutation is cell autonomous, and the contribution of interorgan interactions. For highly conserved cell-autonomous mechanisms (e.g., the cell cycle, genome stability cilia), the choice of lower-order model organisms may facilitate the rapid dissection of

components of the developmental and cellular pathway. For complex behavioral phenotypes or later-evolved genetic networks (e.g., the regulation of skeletal development), higher-order mammalian models may be required. Ultimately, the analysis must take into account gene–gene and gene–environment interactions and the early versus the late effects of the target gene. The availability of genomic information and molecular tool kits for gene targeting and replacement has rapidly accelerated the generation of loss- and gain-of-function models. A major goal for further facilitating the translation of information between species will be approaches for rapidly introducing single nucleotide variants into the model genomes so that an allelic series can be quickly obtained. Another important goal would be to extend the ability to introduce loss- and gain-of-function alleles into the germline of larger-animal models. Ultimately, if the pace of generating models can be further accelerated, a bottleneck will still remain in the characterization of resulting phenotypes. Doubtlessly, success with this challenge will still depend on old-fashioned hard work, intuition, insight, and some degree of luck. Ultimately, the ability to begin to integrate global methods of phenotypic analyses into mechanistic hypotheses that are testable will be critical in completing the loop of translational research between humans and model organisms (Figure 7.1).

The goal of modeling human disease is to enable the testing of hypotheses involving structure and function. These may be deduced from the study of genotype–phenotype correlations in human disease. Translation from human to model organism study should result in the formulation of a genetic and biochemical pathway that both explains the phenotype and generates testable predictions. The translation of mechanistic information back to humans would then involve the testing of these predictions in humans. In closing this loop, we should gain information about a basic developmental process as well as the consequence of dysregulation of this process in humans. The goal of clinical translation would be to develop methodologies to measure and ultimately rescue this pathway.

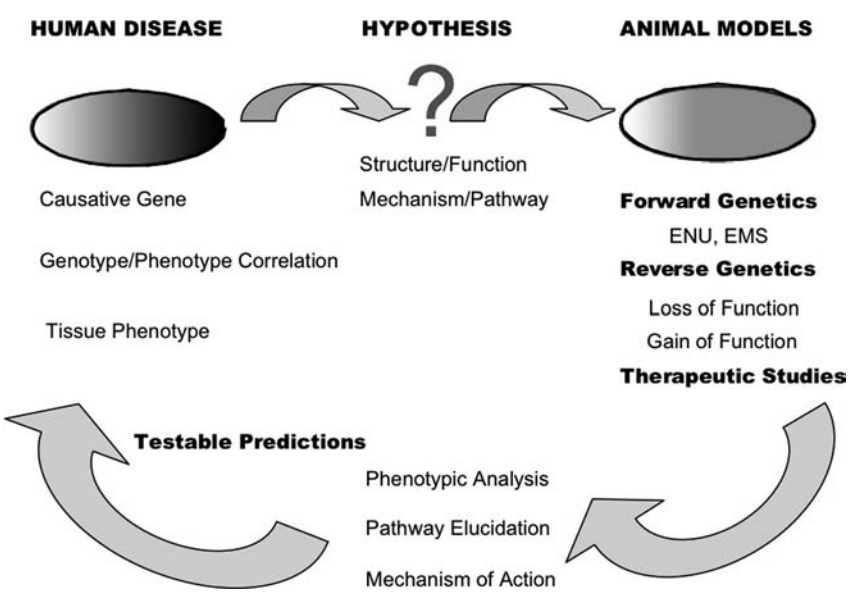

FIGURE 7.1 **The loop of translational research between human and model organisms.** (See color insert).

SUMMARY

- Developmental defects can stem from structural metabolic defects or exposure to environmental agents. Model organisms are indispensable tools for understanding the mechanisms of complex biologic pathways and human diseases.
- The choice of model organism needs to consider the nature of the studied pathway, whether the mutation is cell autonomous, and the contribution of interorgan interactions.
- As a result of the availability of genomic information, a powerful genetic toolkit, and the relative conservation of organogenesis, the mouse model has been the most used for studying human developmental disorders.
- Factors that affect the degree of phenotypic correlation between mice and humans include the following: 1) differences in the dosage sensitivity of the mutation and the affected pathway; 2) differences in redundant pathways and genes; 3) genetic and epigenetic modifiers specific for inbred strains of mice versus outbred humans; 4) true divergent function of the target gene in mouse versus humans; and 5) differential effects seen early versus later in development.
- Comparative human and model organism studies should facilitate a translational loop that enables the elucidation of pathogenic mechanisms and testable predictions that can be validated back in the human condition.
- Technological advances that will accelerate comparative studies include the ability to rapidly generate single nucleotide substitutions in the germ cell, high-throughput methods, and the associated informatic tools to characterize tissue phenotypes on a molecular level.

GLOSSARY OF TERMS

Choreoathetosis
Sudden involuntary movements of the limbs and the facial muscles.

Hyperuricemia
A high level of uric acid in the blood.

Organic acidemia
A class of inherited metabolic disorders that lead to the accumulation of organic acids in biologic fluids (blood and urine).

Pleiotropic effect
A single gene that produces multiple phenotypic traits.

Skeletal dysostosis
The abnormal formation of bone caused by the lack of proper ossification.

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Model organisms
http://www.ncbi.nlm.nih.gov/About/model/
Saccharomyces Genome Database
http://www.yeastgenome.org/
Wormbase *(C. elegans)*
http://www.wormbase.org/
Flybase (Drosophila)
http://www.flybase.org/
Mouse genome informatics
http://www.informatics.jax.org/

II

EARLY EMBRYOLOGY, FATE DETERMINATION, AND PATTERNING

8
GERM LINE DETERMINANTS AND OOGENESIS

KELLY M. HASTON and RENEE A. REIJO PERA

Institute for Stem Cell Biology and Regenerative Medicine, Stanford University, Palo Alto, CA

INTRODUCTION

Germ cells are the only cells in the body that are destined to pass genetic information from one generation to the next; by contrast, somatic cells give rise to cells of the body that are ultimately destined to die. Thus, the allocation (also called *specification*) of germ cell versus somatic cell fate is of primary importance to all species; it occurs very early in embryo development (Saffman and Lasko, 1999). Despite the critical importance of germ cell development to all species, however, two divergent methods of germ cell specification and maintenance are apparent in animals (Saffman and Lasko, 1999; Houston and King, 2000; Wylie, 2000). First, in nonmammalian species, germ cell fate is determined by the inheritance of *germ plasm,* which is microscopically distinct oocyte cytoplasm that is particularly rich in RNAs and RNA-binding proteins and that segregates with cells destined to become germ cells (Saffman and Lasko, 1999; Houston and King, 2000; Wylie, 2000). By contrast, in mammalian species, both male and female germ cells are specified independently of germ plasm via inductive signaling from neighboring cells (Lawson and Hage, 1994; Tam and Zhou, 1996; Lawson et al., 1999; McLaren, 1999; Ying, 2000; Ying et al., 2001; Yoshimizu, 2001). Both modes of specification are discussed in more detail below. We then move on to discuss the migration and maturation of gametes, with the later discussion particularly focusing on oogenesis.

I. GERM CELL SPECIFICATION

A. Germ-Plasm–Dependent Specification of the Germ Cell Lineage

Specialized cytoplasm, called *germ plasm,* is found in diverse nonmammalian species that include *Caenorhabditis elegans* (nematodes), *Drosophila melano-*

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gaster (flies), *Xenopus laevis* (frogs), and *Danio rerio* (fish) (Wylie, 2000; Zhou and King, 2004). The germ plasm is observed in oocytes and cleavage-stage embryos; it is microscopically dense; and it is enriched greatly in RNAs, RNA-binding proteins, mitochondria, and ribosomes (Wylie, 2000; Zhou and King, 2004; see Figure 8.1). Germ plasm is referred to by different names in different species; it is referred to as *P granules* in *C. elegans, pole plasm* in *D. melanogaster,* and simply as *germ plasm* in *X. laevis*. Nonetheless, several common properties define germ plasm. First, in organisms that specify germ cell fate via germ plasm, the decision to segregate the germ cell from somatic lineages occurs before gastrulation. Second, the germ plasm or germline granules segregate at all times with cells of the germ cell lineage, in both embryonic and postembryonic development (see Figure 8.1). Indeed, germ plasm plays a determinative role: cells that inherit germ plasm develop as germ cells, whereas, in the absence of germ plasm, germ cells do not develop. Finally, as noted by several reviewers, despite the differences between the two processes of germ cell allocation, a number of factors that retain their function in germ cell development are highly conserved between organisms that specify germ cells via germ plasm and those that do not. For example, in Drosophila, the disruption of genes such as *Oskar, Vasa, Tudor, Germ cell-less,* and *Aubergine* results in the lack of a germ line (Santos and Lehmann, 2004). These genes function to assemble the germ plasm, in which the highly conserved interacting RNA-binding proteins *Pumilio* and *Nanos* are localized. These proteins in turn repress translation and thus indirectly silence gene transcription in nascent germ cells (Lin and Spradling, 1997; Forbes and Lehmann, 1998; Parisi and Lin, 1999; Santos and Lehmann, 2004). In *Pumilio* and *Nanos* mutants, nascent germ cells may divide prematurely, migrate abnormally, or subsequently die during early embryo development (Jaruzelska et al., 2003; Tsuda et al., 2003; Santos and Lehmann, 2004). Recently, the homologues of these and other germ plasm components have been identified in mammalian germ cells, and, in many cases, these are required for germ cell development, despite the fact that, in mammals, germ cells are not specified via germ plasm in the oocyte and cleavage-stage embryos.

B. Germ-Plasm–Independent Germ Cell Specification (Inductive Signaling)

Initially, in mice, a founder population of approximately 45 primordial germ cells (PGCs) is formed (Chiquoine, 1954; Ginsburg et al., 1990). Fate-mapping studies have been used to examine germ cell specification in mammals, and they have revealed that germ cells are specified in the proximal epiblast in mice (Tam and Zhou, 1996; see Figure 8.2) in response to signals from the neighboring extraembryonic ectoderm, particularly *Bmp4* signaling (Fujiwara et al., 2001; see Figure 8.2). However, it is notable that the proximal epiblast is not predestined to a germ cell fate, because transplantation of the distal epiblast to contact the extraembryonic ectoderm also results in germ cell formation (Tam and Zhou, 1996). Furthermore, the fate of proximal epiblast cells is ultimately to form both germ cells and extraembryonic mesoderm. Thus, it is likely that the extraembryonic ectoderm provides one of the first signals for germ cell specification in the epiblast. Then, a second as yet uncharacterized signal must be required to distinguish extraembryonic mesoderm from germ cells. Germ cells are definitively recognized after gastrulation, at 7.2 days post coitum, as an extraembryonic cluster of cells at the base of the allantois that express tissue

nonspecific alkaline phosphatase, *Oct4,* and *Stella* (Chiquoine, 1954; Scholer et al., 1990; Scholer et al., 1990; Saitou et al., 2002; see Figure 8.2). Notably, although epiblast cells migrate through the primitive streak during gastrulation, the physical act of migration does not appear to be necessary for defining germ cell versus somatic cell fates (Ying et al., 2001; Yoshimizu, 2001; Pesce et al., 2002).

The search for molecules that are required for the specification of germ cells in mammals has been intriguing. Early studies suggested that perhaps a gene called *Stella* may be a determinant of the germ cell lineage (Saitou et al., 2002). However, disruption of the mouse *Stella* gene indicated that the gene encoded a factor that was required for embryo growth; indeed, embryos derived from oocytes that are null for *Stella* do not develop (Payer et al., 2003; Bortvin et al., 2004). Subsequently, Ohinata and colleagues (2005) identified the *Blimp1* gene as a critical determinant of the germ cell lineage. Although the *Blimp1* gene encodes a transcriptional repressor that is widely expressed during development, its function was shown to be required for the establishment of the primordial germ cell population (via repression of somatic *Hox* genes) and for the subsequent migration and proliferation of germ cell populations (Ohinata et al., 2005; see Figure 8.2).

II. CONSERVED GENES IN ORGANISMS THAT SPECIFY GERM CELLS VIA GERM PLASM AND INDUCTIVE MECHANISMS

In the description above, the specification of germ cells via germ plasm and via inductive signaling was contrasted. However, as the DNA sequences of multiple organisms have been assembled and reproductive biologists have probed gene function across species, it has become clear that many key genes that function in establishing and maintaining germ cell populations are conserved. In particular, family members of genes such as *Vasa, Pumilio, Nanos*, and *Deleted in AZoospermia (DAZ)* have homologs in diverse organisms.

A. Vasa

Homologs of the *Vasa* gene family encode RNA-binding proteins of the DEAD-box helicase family, which are specifically expressed in germ cells in all animals examined. The *Vasa* gene was first identified as a maternal-effect gene that is required for the proper establishment of abdominal segments and for the formation of the pole cells in Drosophila (Schüpbach and Wieschaus, 1986). Subsequently, the gene was also shown to function in oogenesis (Styhler et al., 2002). Studies in diverse species have verified that this gene has a function in germ cell development; however, the phenotypes may differ from species to species. For example, in mice, the disruption of *mouse Vasa homolog* leads to the meiotic arrest of male germ cells, whereas there is a reduction in premeiotic germ cells in female flies and an arrest in the pachytene stage of oogenesis in nematodes (Tanaka et al., 2000; Kuznicki et al., 2000; Styhler et al., 2002).

B. Pumilio and Nanos

The *Pumilio* and *Nanos* genes are among the most well-characterized genes in invertebrates. In Drosophila, these genes encode interacting proteins that are

required for the formation of nascent germ cells and for the establishment of the anterior–posterior axis (Kobayashi et al., 1996; Wreden et al., 1997; Asaoka et al., 1998; Asaoka-Taguchi et al., 1999; Deshpande et al., 1999). Then, later in development, these genes may be required for oogenesis as well (Forbes and Lehmann, 1998). In *C. elegans,* there are numerous members of the *Pumilio, Fbf,* and *Nanos* gene families (Subramaniam and Seydoux, 1999; Wickens et al., 2002; Subramaniam and Seydoux, 2003). Where studied, these homologs also function in gametogenesis, with some homologs encoding proteins that interact to promote the spermatogenesis-to-oogenesis switch and others required for the incorporation of germ cells into the gonad or the progression of germ cell development through meiosis (Kraemer et al., 1999; Subramaniam and Seydoux, 1999; Crittenden et al., 2002; Subramaniam and Seydoux, 2003).

Pumilio and *Nanos* homologs have also been identified in mammals, including humans (Rongo et al., 1997; Castrillon et al., 2000; Tanaka et al., 2000; Mochizuki et al., 2001; Jaruzelska et al., 2003; Moore et al., 2003; Tsuda et al., 2003). Indeed, a number of factors that interact in invertebrate germ cells have also been shown to interact in vertebrates, including the *Pumilio* and *Nanos* proteins (Jaruzelska et al., 2003; Moore et al., 2003; Tsuda et al., 2003). Moreover, the loss of function of some homologs results in infertility; in particular, the loss of function of *Nanos2* results in the defective development of male germ cells, and the loss of *Nanos3* function results in the impaired maintenance of PGCs during migration in both sexes (Tsuda et al., 2003). Other known vertebrate *Nanos* homologs include *Xcat-2* in Xenopus and *Nos1* and *Nos2* in zebrafish; the products of these genes have been shown to localize to germ plasm in these species, and zebrafish *Nos1* has been shown to be required for PGC migration and survival (Mosquera et al., 1993; Koprunner et al., 2001).

C. Deleted in AZoospermia

Human *DAZ* was identified in a screen for Y chromosome genes that cause azoospermia (or the production of few or no germ cells) when deleted in men (Reijo et al., 1995; Reijo et al., 1996). Subsequently, autosomal homologs called *Deleted in AZoospermia-Like* (*DAZL)* were identified in mice and humans, and they were also shown to be expressed only in germ cells (Cooke et al., 1996; Reijo et al., 1996; Yen et al., 1996; Menke et al., 1997). Finally, a third homolog called *boule* was identified as a meiotic regulator. It was hypothesized to be the ancestral member of this family, because this gene is conserved in both vertebrates and invertebrates (Eberhart et al., 1996; Xu et al., 2001; Xu et al., 2003). Notably, all members of the *DAZ* gene family encode RNA-binding proteins that contain a highly conserved RNA recognition motif, and they may bind several different RNAs during the development of male and female germ cells pre- and postmeiotically (Houston et al., 1998; Venables and Eperon, 1999; Houston and King, 2000; Tsui et al., 2000; Tsui et al., 2000; Venables et al., 2001; Jiao et al., 2002; Collier et al., 2005; Fox et al., 2005; Reynolds et al., 2005). The loss of function of members of this gene family demonstrates that they function in germ plasm, in meiosis, and postmeiotically in different organisms (Ruggiu et al., 1997; Houston et al., 1998; Houston and King, 2000; Karashima et al., 2000; Saunders et al., 2003; Dann et al., 2006). In addition, studies of *DAZL*

function in humans have demonstrated that, despite their key role in germ cell development in numerous species genes, the *DAZ* and *DAZL* genes are among the most variable in the human genome, with variants that correlate with reproductive parameters in both men and women (Teng et al., 2006; Tung et al., 2006a; 2006b).

III. GERM CELL MIGRATION

After specification and just before or during the early stages of gastrulation, germ cell migration occurs. PGCs migrate out of the embryo proper and reside in extraembryonic tissues until gastrulation is complete. PGC migration in the mouse occurs between embryonic days 7.5 and 13.5, when the PGCs travel through the developing gut to become incorporated into the primitive gonad (see Figure 8.2). The migration of PGCs is a multistep process during which the cells migrate through different tissue types and environments (Anderson et al., 2000; Molyneaux et al., 2001; Molyneaux and Wylie, 2004; Santos and Lehmann, 2004). Both migratory and survival signals appear to be required during this period for successful PGC development. After embryonic day 7.5, there is a distinct population of cells that appear to have a germ-cell–specific gene-expression profile. Several factors are involved in germ cell survival as PGCs progress along their migratory route. For example, the gene *Dead end,* which is required for the initiation of migration in zebrafish, appears to act in the mouse as a survival factor. Mice with mutations in this gene display a decrease in PGC number by embryonic day 8.0 and have increased testicular germ cell tumors detected after birth (Youngren et al., 2005). Another gene, *Tiar,* encodes an RNA-binding protein that is necessary for PGC survival and that has been implicated as a regulator of apoptosis. The *Tiar* protein is required on embryonic day 11.5 during migration from the hindgut to the genital ridge, and mice lacking this protein fail to develop oogonia or spermatogonia (Beck et al., 1998). Furthermore, the growth factor *Fgf2,* its receptor *Fgf2-IIIb,* and other genes (e.g., the antiapoptotic gene *Bax)* are required for the survival of germ cells during the migration from the hindgut to the genital ridge (Sette et al., 2000; Stallock et al., 2003; Takeuchi et al., 2005).

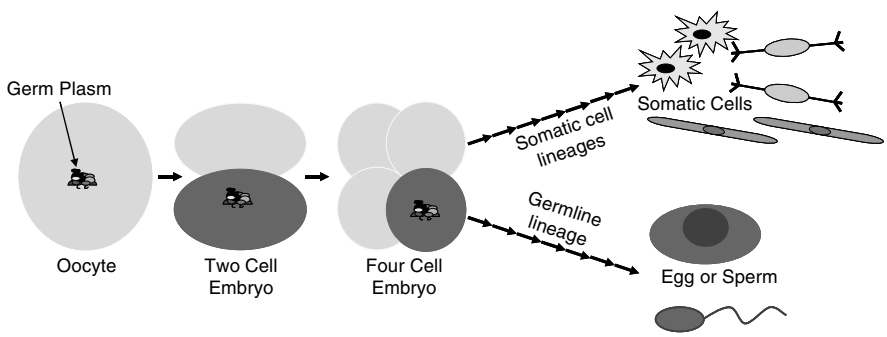

FIGURE 8.1 The pregastrulation specification of germline fate through the inheritance of germ plasm. Oocytes from species with predetermined germ cell specification contain a microscopically dense complex that is enriched in RNAs, RNA-binding proteins, mitochondria, and ribosomes. During the earliest embryonic cell divisions, germ plasm segregates to cells that will eventually give rise to the germ cell lineage. Cells that do not contain germ plasm give rise to somatic lineages. (See color insert.)

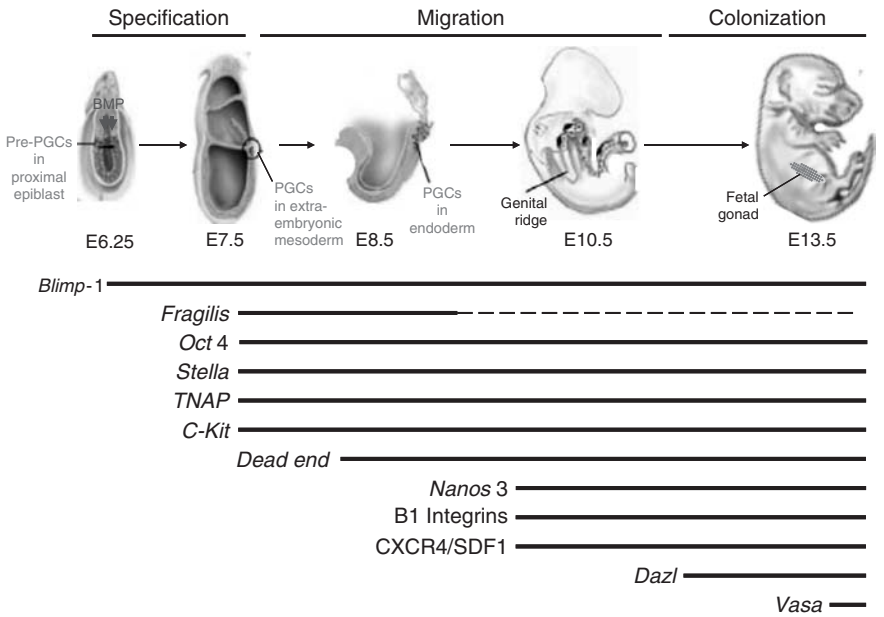

FIGURE 8.2 **Primordial germ cell (PGC) development in the mouse.** Stages of PGC development during the postfertilization mouse embryo, from embryonic days 6.25 to 13.5. Displayed below the figure is the PGC-specific expression of genes that are either required for or diagnostic of the specified stage of PGC development. PGCs are displayed as red dots, which are specified in the proximal epiblast (blue) at embryonic day 6.25 in response to bone morphogenetic protein signaling from the extraembryonic tissues (green arrows). PGCs then migrate into the extraembryonic mesoderm by embryonic day 7.5. By embryonic day 8.5, they have entered the definitive endoderm, and they begin their migration down the developing hindgut. They colonize the primitive fetal gonad between embryonic days 11.5 and 13.5. (Modified from National Institutes of Health: *Stem cells: scientific progress and future research directions* [Web site]: stemcells.nih.gov/info/scireport/2001report.htm. Accessed December 11, 2006. See color insert.)

Although some survival signals required during the earliest stages of PGC migration have been identified, early migratory steps are poorly understood, and few factors have been identified that clarify what triggers these events. However, several mediators during the later stages of migration involving the hindgut-to-genital-ridge transition have been resolved. On approximately embryonic day 8.0, the PGC population migrates out of the extraembryonic mesoderm and enters the embryonic (definitive) endoderm. Although they display motile behavior both *in vivo* and *in vitro*, they do not actively migrate at this point; rather, they are carried along with the endoderm as the hindgut invaginates between embryonic days 8.5 and 9.0, and, by embryonic day 9.0, PGCs are in the hindgut epithelium (Anderson et al., 2000; Molyneaux et al., 2001; Molyneaux and Wylie, 2004). Between embryonic days 9.0 and 9.5, PGCs emerge from the hindgut and migrate into the developing genital ridge. On embryonic day 10.5, the PGCs are moving toward the genital ridges from divergent sites, and PGCs that do not reach the genital ridge undergo apoptosis.

The migration of PGCs from the hindgut to the genital ridge is an essential step in germ cell development. Factors involved in this step include the RNA-binding protein *Nanos3* (described previously), the adhesion molecules β1 integrins, the forkhead/winged helix transcription factor *Foxc1,* and the G-protein–coupled receptor CXCR4 and its ligand SDF-1 (Anderson et al.,

1999; Anderson et al., 2000; Ara et al., 2003; Molyneaux et al., 2003; Tsuda et al., 2003; Mattiske et al., 2006). In particular, *Nanos3* is expressed in PGCs by embryonic day 9.5, and the loss of this protein leads to a decline of PGC number by embryonic day 11.5 in both sexes (Tsuda et al., 2003; see Figure 8.2). Similarly, the loss of β1 integrins leads to a decline in PGC number before they reach the genital ridges, although the mechanism for this reduction is not understood (Anderson et al., 1999). *Foxc1* mutants also result in the failure of PGCs to exit the hindgut (Mattiske et al., 2006). Finally, the G-protein–coupled receptor CXCR4 is expressed on the surface of the PGCs, and both the body wall mesenchyme and the genital ridges express SDF-1; the loss of either the receptor or the ligand leads to the failure of PGCs to reach the genital ridge, and this is followed by cell death of ectopic PGCs in the hindgut (Ara et al., 2003; Molyneaux et al., 2003; see Figure 8.2). Although this is not an exhaustive list, it is clear from this brief overview of factors involved in hindgut-to-genital-ridge migration that both PGC autonomous and nonautonomous interactions are required for both the earlier and later stages of PGC survival and migration. By embryonic day 11.5, the majority of the PGCs (~25,000) are in the genital ridges, where they are becoming nonmotile and beginning to aggregate into sex-specific organization with the somatic tissue (Anderson et al., 2000; Molyneaux et al., 2001; Molyneaux and Wylie, 2004; see Figure 8.2).

IV. GERM CELL SEX DETERMINATION

After the germ cells reenter the embryo and migrate into and invade the genital ridges, they will colonize, proliferate mitotically, complete the process of resetting the genomic imprints (described later) by erasure (on embryonic days 9.5 to 11.5), and differentiate as either male or female germ cells (Gomperts et al., 1994; Hajkova et al., 2002). Before entry into the gonad, the development of male and female germ cells has been indistinguishable; subsequently, however, germ cell development in the male and female gonads will diverge (Swain, 2006). Germ cells that colonize an ovary will enter the first meiotic prophase and become oogonia at approximately embryonic day 13.5, whereas those that colonize the testis will not progress to meiotic prophase and will instead mitotically divide to form a pool of spermatogonia that may replicate or differentiate throughout the life of the male (Swain, 2006).

Several reports document early events in the sex determination of mammalian germ cells (Menke et al., 2003; Bowles et al., 2006; Koubova et al., 2006). The differentiation of cells to female or male germ cells occurs independently of sex chromosome composition and instead is dependent on gonadal sex (Swain, 2006). An early molecular marker of female sexual differentiation is the expression of the *Stra8* gene, which is expressed in an anterior-to-posterior wave in germ cells in the ovary between embryonic days 12.5 and 16.5 (Menke et al., 2003). Coincident with or just after *Stra8* expression, the downregulation of the *Oct4* gene (a marker of germ cells that is most highly expressed before meiosis) and the upregulation of *Dmc1* (a gene that encodes a meiotic protein) ensues (Menke et al., 2003). These observations further suggest that local signals may regulate the sexual differentiation of the germ cells in an anterior-to-posterior fashion and promote meiotic entry (for more information about meiosis is provided later in this chapter).

Early studies suggested that both male and female germ cells are inherently programmed to enter meiosis in the fetal gonad and that the inhibition of meiosis via a hypothesized "meiosis-inhibiting factor" must be a critical event in male germ cells (McLaren and Southee, 1997). Moreover, data indicated that one of the factors implicated in regulating the sex-specific timing of meiotic initiation in mice is retinoic acid, which may act by regulating the expression of *Stra8* (Koubova et al., 2006). Furthermore, in the male, the identity of a factor that may act as the meiosis-inhibiting factor was suggested with the disruption of a gene that encodes the retinoid-degrading enzyme CYP26B1; testis in which this gene is disrupted possess germ cells that enter meiosis precociously, as is expected of germ cells in the ovary (Bowles et al., 2006). Together, this work provides the first molecular explanation of how germ cell sexual differentiation is regulated in mammals.

V. GENOMIC IMPRINTING

Genomic imprinting is another fundamental property of mammalian germ cells. Epigenetic modifications to the genome (typically by DNA methylation) result in the expression of genes from only one of the two parental chromosomes. The "instructions" regarding gene expression are established in the parental germ cells via differential methylation of the DNA. Methylation predominantly occurs on the cytosine residue of CpG dinucleotides, which typically cluster together to form "CpG islands" (Reik and Walter, 2001). The establishment of these imprints in germ cells is essential for fetal, placental, and behavioral development (Reik and Walter, 2001). The misregulation of imprinted genes, which leads to biallelic expression, has been implicated in growth and neuronal disorders in multiple mammalian species, including humans (Allegrucci et al., 2004; Kelly and Trasler, 2004; see Chapter 5).

Interestingly, genomic imprints are altered throughout the life cycle of the organism (Reik and Walter, 2001; Hajkova et al., 2002). Upon fertilization, imprinting is maintained through the replication and segregation of chromosomes during development. Although the mechanism for the maintenance of specific regions of DNA is not fully understood, one of the five mammalian DNA methyltransferases, *Dnmt1,* has been identified as a maintenance methyltransferase that is essential for the maintenance of methylation during DNA replication (Bestor, 2000). As germ cells develop in the new organism, genomic imprints in PGCs are erased by a wave of demethylation, which occurs as they are arriving at the primitive gonadal ridge. It is at this stage that an active erasure of methylation at the imprinted regions occurs by an unidentified demethylation agent.

Imprints are then reestablished in a sex-specific manner in the PGCs through de novo methylation by *Dnmt3a, Dnmt3b,* and *Dnmt3L* (Okano, 1999; Bourc'his et al., 2001; Hata et al., 2002). The imprints are established in germ cells as they mature into sperm or eggs at different time points for female versus male imprints. Imprints in prospermatogonia are imposed before they enter meiosis, whereas imprints in oocytes are reestablished later, at different stages of oogenesis, in a gene-specific manner (Bestor and Bourc'his, 2004). The reestablishment of sex-specific imprints is an essential step in gametogenesis, which completes the cycle toward a mature germ cell that is competent to give rise to viable offspring.

VI. MEIOSIS

A. General Properties of Meiosis

Meiosis is defined by a series of stages with characteristic landmark events, and it is highly conserved across species (Kleckner, 1996). Prophase I is a defining stage of meiosis that encompasses many unique features, including the formation of the synaptonemal complex, the pairing of homologous chromosomes, and the formation of chiasmata between homologs. These features are common to almost all species, and they are in place to ensure that homologous chromosomes pair and remain together until the first meiotic division (Kleckner, 1996). Prophase can be divided into four stages: leptotene, zygotene, pachytene, and diplotene. DNA replication begins in the preleptotene to leptotene stages, and sister chromatids begin to condense. Then, during zygotene, sister chromatids synapse along their length and form lateral elements that contain synaptonemal complex proteins *(SCPs)*, such as *SCP2* and *SCP3*. At pachytene, synaptonemal complex formation is complete, and recombination nodules that contain proteins such as *MLH1* are clearly visible. Finally, at the diplotene stage, homologous chromosomes begin to separate, and only the chiasmata (the sites of the recombination machinery) hold the chromosomes together. Meiotic division continues to progress in an orderly fashion from meiosis I to meiosis II, unless errors in the recombination or chromosomal segregation machinery trigger arrest at one of two checkpoints, either during prophase or at the metaphase–anaphase transition (Roeder, 1997; Roeder and Bailis, 2000). Then, after meiosis, germ cells continue to develop through a process called *spermiogenesis* in males, during which they become mature elongated spermatids by the compaction of their chromatin into the sperm head, by the production of other sperm components (e.g., the flagellar tail), and by oocyte maturation in females (Hunt and Hassold, 2002).

There are many genes involved in the initiation of and the progression through meiosis, and this has been a subject of intense study for several decades in many different organisms (Baker et al., 1976; Kleckner, 1996; Smith and Nicolas, 1998; Roeder and Bailis, 2000; Hunt and Hassold, 2002). The disruption of the function of meiotic genes generally leads to meiotic arrest and subsequent apoptosis of the germ cells or aneuploidy (Lahn and Page, 1997).

B. Errors in Meiotic Chromosome Segregation

Aneuploidy arises during meiosis I or II by nondisjunction or the premature separation of sister chromatids, and it is a rare event in most organisms. For example, rates of aneuploidy in meiotic cells have been reported as 1 in 10,000 cells for *Saccharomyces cerevisiae,* 1 in 6000 cells for *D. melanogaster,* and approximately 1 in 100 to 1 in 200 for mice (Hassold and Hunt, 2001). Surprisingly, the rate of aneuploidy in humans may be as high as 1 in 10 to 1 in 30, depending on factors such as age and sex (Hassold and Hunt, 2001).

Aneuploidy is detected in approximately 5% of clinically recognized pregnancies. However, in general, most aneuploidies are eliminated early in gestation. Among fetal deaths occurring between about 6 to 8 weeks' and 20 weeks' gestation, about 35% are trisomic or monosomic; this rate decreases to about 4% among stillbirths (fetal deaths occurring between about 20 weeks' gestation and term) and to about 0.3% among newborns (Hassold

and Hunt, 2001). The most common abnormalities in both stillbirths and newborns are trisomy 21 and sex chromosome trisomies (47,XXX; 47,XXY; 47,XYY). The overall rate of 5% aneuploidy in all human conceptions is likely an underestimate, because it does not include an analysis of undetected pregnancies during the first few weeks of gestation (Hassold and Hunt, 2001).

Aneuploidy is also common in human gametes. Approximately 2% of human sperm are aneuploid, and 20% to 25% of oocytes are aneuploid (depending on age) (Hassold and Hunt, 2001). Studies of the origin of aneuploidy over the past decade have revealed that maternal errors predominate among almost all trisomies, with paternal errors accounting for nearly 50% of 47,XXYs and trisomy 2.

VII. OOGENESIS

Oogenesis is the meiotic division of a diploid oocyte into a haploid ovum (egg). The process of oogenesis is dependent on the development of an ovarian follicle (folliculogenesis), which is the formation of the somatic cells surrounding the developing oocyte that will become the functional unit of the ovary

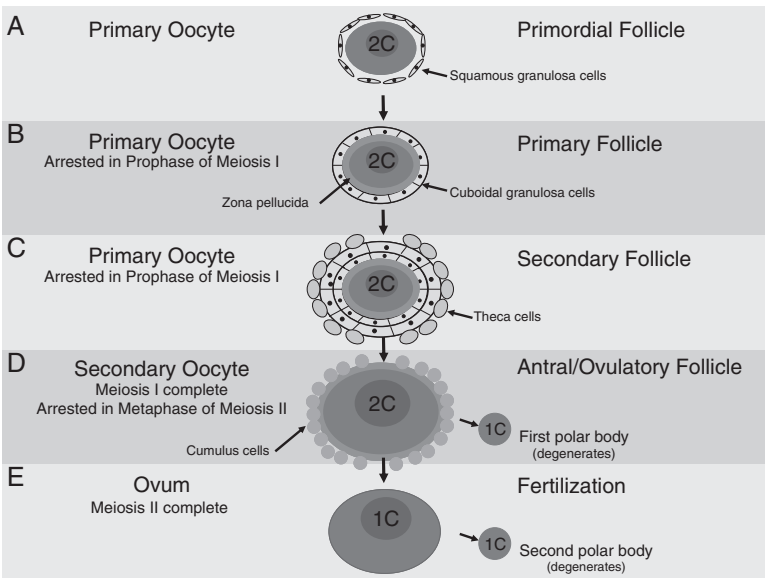

FIGURE 8.3 **Oogenesis and folliculogenesis.** Oogenesis and folliculogenesis are intertwined processes that form the functional unit of the ovary. **A,** Both processes begin when the primary oocyte, which at this point is diploid (2C), becomes surrounded by squamous granulosa cells to make up the primordial follicle. **B,** The primary oocyte enters meiosis and becomes arrested during the prophase of meiosis I as the granulosa cells of the follicle transition from squamous to cuboidal to form a primary follicle. During this process, the zona pellucida or egg coat is formed around the primary oocyte. **C,** The primary oocyte maintains its meiotic arrest, whereas the primary follicle develops into the secondary follicle through the formation of multiple layers of granulosa cells surrounded by theca cells. **D,** After the formation of the antral follicle, the oocyte is ovulated; it is surrounded by cumulus cells, and it completes meiosis I, extruding the first polar body, which will degenerate. It is now a secondary oocyte, and it is arrested during the metaphase of meiosis II. **E,** Finally, upon fertilization, meiosis II is completed with the extrusion of the second polar body, which will also degenerate. This will leave an ovum, which is haploid (1C) and fully competent to support embryonic growth. (See color insert.)

(see Figure 8.3). The interaction between the oocyte and surrounding follicular structure is essential for the correct timing of events during oogenesis (Mehlmann, 2005). These events include the maintenance or release of the two periods of meiotic arrest that the oocyte experiences during its development, which occur during the prophase of meiosis I and the metaphase arrest of meiosis II. Because of the critical nature of the oocyte/follicle interaction, folliculogenesis is highly regulated by endocrine, paracrine, and autocrine factors (Roy and Matzuk, 2006).

In vertebrates, oogenesis begins during embryogenesis, after the primordial germ cells migrate into the primitive ovary. These cells now complete mitotic divisions and become primary oocytes by entering meiosis and arresting during the diplotene phase of meiotic prophase I (see Figure 8.3, B). The first step in follicular development is dependent on the expression of a basic helix-loop-helix transcription factor, factor in the germ line α *(Figlα)*, by the primary oocytes, which triggers the formation of a primordial follicle that consists of a single layer of flat, squamous, pregranulosa cells around the oocyte. *Figlα* is essential in the formation of the primordial follicle, and *Figlα*-null mice are infertile and do not have primordial follicles (Liang et al., 1997). In mice, the majority of primary oocytes are in primordial follicles within 1 to 2 days of birth, whereas, in humans, primordial follicles form at 19 weeks' gestation (Choi and Rajkovic, 2006).

After primordial follicles are established, further follicle development involves the periodic recruitment of a subset of the follicles into a maturation cycle either postnatally (in mice) or at puberty (in humans). Although the mechanism of follicle recruitment is not well understood, the process begins with the transition of a primordial follicle to a primary follicle, at which time the granulosa cells surrounding the oocytes now undergo a squamous-to-cuboidal shape change and begin to undergo proliferation (see Figure 8.3, A and B). The oocyte-specific expression of the homeobox transcription factor *Nobox* is required for the primordial-to-primary transition to occur, and *Nobox* mutants display a block in oocyte development and infertility; they also have a decreased number of somatic cells surrounding the oocytes (Rajkovic et al., 2004). The primary oocyte also continues to express *Figlα,* which drives the expression of the zona pellucida genes during primary follicle formation (Liang et al., 1997). The zona pellucida gene products *ZP-2* and *ZP-3* function at this time to form the zona pellucida oocyte coat that will be needed later, during oocyte maturation and fertilization. Also important in the developing crosstalk between the oocyte and its surrounding somatic follicle is the expression by granulosa cells of the forkhead transcription factor forkhead box L2, *FOXL2,* which is required for the squamous-to-cubiodal transition of the granulosa cells (Choi and Rajkovic, 2006).

The primary follicle then develops into a secondary follicle through the formation of two or more layers of cuboidal granulosa cells surrounding the primary oocyte, and this is followed by the development of theca cells around the granulose cell layer (see Figure 8.3, B and C). This process is dependent on the oocyte-specific expression of growth differentiation factor 9 *(Gdf9)* and bone morphogenetic protein 15 *(BMP-15)*. Both are members of the TGF-β superfamily of secreted proteins, and both are required for the primary-to-secondary transition. Without the oocyte-specific expression of these genes, the development of the somatic cells surrounding the oocytes fails, which will lead to the cell death of the oocyte (Carabatsos et al., 1998). Within

the secondary follicles, the primary oocytes reach their maximum sizes of ~75 μm and ~100 μm in diameter in mice and humans, respectively (Mehlmann et al., 2004; Mehlmann, 2005), and they are competent to resume meiosis; however, the primary oocyte is still arrested in meiotic prophase I. The G-protein coupled receptor *Gpr3* maintains the meiotic arrest by promoting high levels of cAMP in the oocyte, and *Gpr3*-mutant mice undergo spontaneous oocyte maturation (Mehlmann et al., 2004; Mehlmann, 2005); however, the ligand that activates the receptor has not been identified.

The next step in folliculogenesis is the formation of the antral follicle, which is the formation of a fluid-filled cavity or antrum (see Figure 8.3, D). Furthermore, at this stage, the granulosa layer is divided by the antrum into two separate compartments: the outer layer of cuboidal mural granulosa cells and the cumulus cells, which surround the oocyte (Mehlmann et al., 2004; Mehlmann, 2005). The stages of antral development are dependent on endocrine signals from the pituitary gonadotropin hormone follicle stimulating hormone and luteinizing hormone. Along with triggering the growth and differentiation of the somatic cells surrounding the oocyte, these signals also induce steroidogenic enzyme expression. Growth and steroid production help bring the oocyte to a maturation point during the late antral/preovulatory stage. However, although endocrine signaling has played an important part in this process, crosstalk between the oocytes and the somatic environment is still essential, and this is exemplified by the oocyte-specific transcription factor, *Taf4b*. *Taf4b* is a TATA box-binding protein (TBP)-associated factor that is required for the antral-to-preovulatory transition, and female mice lacking this gene are infertile (Falender et al., 2005).

By the late antral/preovulatory stage, the oocyte responds to a surge in luteinizing hormone, which leads to ovulation through an interaction with luteinizing-hormone receptors on the mural granulosa cells. In frogs and fish, the signal is propagated by the stimulation of steroid hormones (Haccard and Jessus, 2006), but how the mural granulosa cells transmits this signal to either the cumulus cells or the oocyte in mammals is currently unknown. Other factors intrinsic to the follicle are also important during this period. One example in the mouse is the orphan nuclear receptor steroidogenic factor 1, which is expressed by the somatic cells of the follicle. Female mice that lack this gene in their ovary have antral follicles, but they do not ovulate (Pangas and Rajkovic, 2006).

After ovulation proceeds, it promotes a resumption of meiosis, with the concurrent extrusion of the first polar body followed by arrest in meiosis metaphase II (see Figure 8.3, E). Now considered a secondary oocyte and surrounded by cumulus cells, the oocyte is able to undergo fertilization by sperm. Upon fertilization, the oocyte will complete meiosis II, extrude the second polar body, and be fully functional and able to support embryonic development.

VIII. GERM CELL DEVELOPMENT IN HUMANS AND INFERTILITY

Infertility is common among both men and women. Although human reproduction and fertility have been studied for many years, few genes have been identified that contribute to human germ cell production. However, several studies have demonstrated that the age at onset of menopause has a significant genetic component; this property is likely to reflect the quantity and quality

of female germ cells that are formed and differentiated. Family history is a significant predictor of early menopause (menopause at age <47 years), and it is reflected by an increased risk of early menopause in women with affected siblings of approximately sixfold (Cramer et al., 1995). In addition, sibling studies have estimated the heritability of the timing of menopause to be high, and several studies have documented the role of discreet regions of the X chromosome in families with a history of early menopause and in those without a family history (Santoro, 2001; Taylor, 2001; Testa et al., 2001; Laml et al., 2002; Shibanuma et al., 2002; Loffler et al., 2003). In addition, a number of genes may be associated with more rare forms of ovarian failure, including the *FOXL2* gene, which is clearly associated with blepharophimosis ptosis epicanthus inversus syndrome, a genetic condition that is associated with aberrant eye development and female infertility, as well as *EIF-2B* and the follicle-stimulating hormone receptor gene, *FSHR* (Aittomaki et al., 1995; Aittomaki, 1996; Crisponi et al., 2001; Beysen et al., 2004).

Likewise, there are several studies that have investigated the genetic component of sperm production. In these studies, the most common genetic lesions associated with spermatogenic defects are deletions of the Y chromosome, including deletions that encompass the *DAZ* gene, which are associated with azoospermia (no sperm in the ejaculate) and oligozoospermia (<20 million sperm per mL of ejaculate) (Reijo et al., 1995; Reijo et al., 1996; Vogt, 1997). Rare point mutations and polymorphisms are also linked to male infertility, in several genes and at several loci (Cooke et al., 1998; Cooke, 1999; Matzuk and Lamb, 2002). Nonetheless, the genetic basis for the failure of germ cell development in men and women is not yet well understood.

IX. GERM CELL DEVELOPMENT *IN VITRO*

The embryologic period during human embryo development that is equivalent to that of mouse germ cell specification occurs shortly after implantation. Thus, the analysis of human germ cell specification and maintenance *in vivo* has been largely inaccessible to biologic and genetic study. However, recent studies have shown that embryonic stem cells (ESCs) derived from the inner cell mass of the blastocyst before epiblast formation are capable of differentiating into both female and male germ cells *in vitro* (Hubner et al., 2003; Toyooka et al., 2003; Clark et al., 2004; Geijsen et al., 2004; Lacham-Kaplan et al., 2005; Nayernia et al., 2006). Oocyte differentiation from mouse embryonic stem cells (mESCs) was obtained via the spontaneous differentiation of adherent cultures as indicated by the analysis of germ-cell–specific markers such as *Vasa*, *Gdf9,* and *Scp3* and as corroborated by the analysis of morphology and follicular steroidogenic enzyme production (Hubner et al., 2003; Lacham-Kaplan et al., 2005). Similarly, male germ cell differentiation was demonstrated via the differentiation of mESCs into embryoid bodies (EBs) and the analysis of germ-cell–specific markers (Toyooka et al., 2003; Geijsen et al., 2004; Nayernia et al., 2006). In one study, initial differentiation *in vitro* was followed by the coaggregation of PGCs with cells from embryonic day 13.5 gonads and the transplantation of aggregates to adult testis (Toyooka et al., 2003). Remarkably, only PGCs readily formed abundant cells with morphologic characteristics of sperm; the transplantation of mESCs

resulted in the generation of teratomas. In a second study, the authors demonstrated that the imprinting status of genes such as *Igfr2* was diagnostic of PGCs and that haploid male gametes derived from ESCs *in vitro* were capable of fertilizing oocytes and activating their development into blastocysts (Geijsen et al., 2004). Most recently, another group demonstrated that mESC-derived male gametes can generate offspring in mice, thus bringing the work full circle to the ultimate proof of functional gametogenesis *in vitro* (Nayernia et al., 2006).

Like mESCs, human ESCs (hESCs) also appear to possess the ability to contribute to the germ cell lineage as demonstrated by the differentiation of three independently derived hESC lines into EBs and the assessment of germ cell development *in vitro* via the analysis of RNA and protein markers diagnostic of germ cell development (Clark et al., 2004; Clark et al., 2004). Markers examined in studies of mESC differentiation into the germ cell lineage were included in all of the human experiments (Hubner et al., 2003; Toyooka et al., 2003; Clark et al., 2004; Clark et al., 2004; Geijsen et al., 2004). Clearly, the early steps of human germ cell development were efficiently completed *in vitro* (Clark et al., 2004; Clark et al., 2004). Finally, the process of germ cell differentiation *in vitro* has been shown to be responsive to growth factors that are implicated in germ cell development *in vivo*. Previous data (primarily from the mouse) have demonstrated the central role of bone morphogenetic proteins (BMPs) in germ cell specification and maintenance early during development. Initially, as described previously, PGCs arise in the proximal epiblast in response to inductive signaling by BMP proteins (Tam and Zhou, 1996; Fujiwara et al., 2001). The analysis of mice carrying null mutations of the *Bmp4, Bmp7,* and *Bmp8b* genes revealed that all three genes play essential roles in the initial stages of germ cell development (Zhao, 2003). *Bmp4*-null mutants have the most severe defect in germ cell development with a near complete absence of PGCs, whereas *Bmp7* and *Bmp8b* knockouts demonstrated a severe reduction in germ cell numbers, especially when they were homozygous null mutants for both genes (Zhao et al., 1996; Ying, 2000; Ying et al., 2001; Zhao, 2003).

Additional data have demonstrated that the role of BMPs in germ cell specification *in vivo* can be recapitulated *in vitro*. For example, in mouse epiblast explant cultures, both human *Bmp4* and *Bmp8b* proteins have been shown to induce PGC formation (Ying et al., 2001). In other studies, the co-culturing of *Bmp4*-producing cells with mESCs similarly increased the number of PGCs formed; the identity of the cells was confirmed by the subsequent transplantation of the enriched PGCs to the mouse testis and the demonstration of meiotic and postmeiotic differentiation to elongated spermatids (Toyooka et al., 2003). These studies strongly suggest that recombinant BMPs can induce germ cell differentiation *in vitro*, particularly from mESCs.

Other studies have subsequently demonstrated that the differentiation of hESCs to germ cells is also responsive to BMPs (Kee et al., 2006). The addition of recombinant human *Bmp4* increased the expression of the germ-cell–specific markers, *Vasa* and *SYCP3,* during the differentiation of hESCs into EBs (Kee et al., 2006). In addition, *Bmp7* and *Bmp8b* showed additive effects on germ cell induction when they were added together with *Bmp4*. Finally, it was shown that the addition of BMPs to differentiating embryonic stem cells also increased the percentage of cells that stained positively for *Vasa* (Kee et al., 2006).

CONCLUSIONS

Each of the processes described above—germ cell specification, migration, sexual differentiation, the erasure and establishment of sex-specific genomic imprinting, meiosis, and morphogenesis—must be precisely executed to form a functional germ cell that can ultimately contribute its genetic information to an embryo and mature offspring. The number of genes required to form a germ cell is likely to be hundreds to several thousand. The functions of only a small subset of genes have been described in any organism; thus, much remains to be learned about the fundamental pathways of germ cell development.

The necessity of probing mammalian germ cell development—including that of humans—is increasingly apparent. This research is particularly responsive to the health concerns of infertile couples who seek assisted reproductive technologies in the hope of achieving biologic parenthood. It would be unfortunate if we did not address the reproductive problems of these men and women, given our progress in the development of tools of analysis and the tremendous contribution of these men and women to the hESC field of study.

Advances in assisted reproductive techniques, reproductive biology, the sequencing of the human genome, and the derivation of hESCs may now allow us to overcome two historically significant limitations in human developmental genetic studies: namely, the inaccessibility of early human development to biologic exploration and the genetic intractability of the human genome during development. Furthermore, the use of hESCs for the *in vitro* derivation of germ cells will potentially circumvent the need for oocyte donation, which is a limiting factor both biologically and ethically. Future research promises to strengthen our basic understanding of the remarkable pathways by which mammalian germ cells develop, to provide useful tools for basic scientists to study human germ cell development, and to contribute to the development of validated diagnostic genetic tests and potential therapeutics for clinical use.

SUMMARY

- Two divergent methods of germ cell specification and the maintenance of early germ cells in animals are evident.
- Many genes are conserved across diverse species that specify germ cells via germ-plasm–dependent and inductive signaling, including the *Vasa, Nanos, Pumilio,* and *DAZ* families of genes.
- In all animal species, germ cells migrate from their site of specification to a position outside of the embryo proper only to subsequently migrate to the gonads after their differentiation.
- Germ cell sex determination occurs after the germ cells have migrated to the nascent gonads, and it occurs independently of chromosomal sex of the germ cell.
- The erasure and reestablishment of sex-specific genomic imprints are diagnostic of the germ cell lineage and integral parts of germ cell development.
- Meiosis constitutes a coordinated set of landmark stages of germ cell development that culminates with the production of a gamete with a haploid genome.

- Meiotic errors are common in some species (e.g., humans) and much less common in others (e.g., invertebrates).
- Infertility, which is characterized by defects in germ cell development, is common among both men and women.
- Germ cells may be differentiated *in vitro* from embryonic stem cells, which allows for an efficient system for the biologic observation of germ cell development.

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GLOSSARY OF TERMS

Genomic imprinting
A phenomenon in which a small subset of genes in the genome are expressed only from one allele according to the parent of origin of the allele. Some imprinted genes are expressed from a maternally inherited chromosome and silenced on the paternal chromosome, whereas other imprinted genes show the opposite expression pattern and are only expressed from a paternally inherited chromosome. The silencing of specific alleles occurs through the methylation of sequences of DNA on a given imprinted gene.

Germ cell
A cell of different stages of development that ultimately will give rise to differentiated sperm or eggs.

Germ cell commitment
The process by which a previously specified germ cell progenitor becomes dedicated to developing only as a germ cell, thereby leading to a restriction in pluripotency.

Germ cell migration
The amoeboid movement of the germ cells toward the developing gonad as a result of internal and external signals, often through several different tissue types or environments. Migrating germ cells are generally maintained in an undifferentiated state, and, in some species, the nascent germ cells proliferate during migration.

Germ cell specification
The acquisition of germline fate, which is in contrast with the somatic cell fate. Germ cell specification is a labile state, and it can be reversible if the cell does not receive further appropriate signals to promote development along the germ cell lineage. There are two divergent methods of germ cell specification in animals: germ-plasm–dependent specification and inductive specification.

Germ plasm
Microscopically visible oocyte cytoplasm that is enriched in RNAs, RNA-binding proteins, mitochondria, and ribosomes. Germ plasm, when present, segregates to cells of the germ cell lineage.

Imprint erasure
The process whereby parental imprints in the diploid germline cells are erased through the removal of the DNA methylation marks. This process is followed by a reestablishment of sex-specific imprints in the germ cells, generally during spermatogonia in males and oocyte maturation in females.

Meiosis
Two rounds of cell division by which germ cells (eggs and sperm) are produced through the division of one diploid cell into four haploid cells. Meiosis is the basis of sexual reproduction, and it only occurs in eukaryotes. The two rounds of cell division are called *meiosis I* and *meiosis II*.

Oocyte maturation
The reinitiating and completion of the first meiotic division, which takes place when oocytes have undergone extensive growth and extruded the first polar body. This is followed by subsequent progression to metaphase in meiosis II.

Oogenesis
The differentiation of female germ cells to form a haploid ovum or egg cell in the ovarian follicle of the ovary. The oocyte divides; one part becomes an ovum, and the other becomes a polar body. There are two major periods of arrest in oogenesis: a fetal arrest during the prophase of meiosis I and a metaphase arrest during meiosis II, which lasts until fertilization occurs.

Primordial germ cell
An undifferentiated precursor germ cell that is set aside from other cells in the developing embryo and that expresses a germ-cell–specific gene profile. Primordial germ cells give rise to oogonia or spermatogonia, depending on the sex of embryo.

Somatic cell
A cell of diverse lineages (extra-embryonic, endoderm, mesoderm, or ectoderm) that will give rise to a differentiated cell of the soma (body) but not to germ cells.

Spermatogenesis
The differentiation of diploid spermatogonia into mature haploid spermatozoa (sperm) cells. In some organisms, spermatogenesis has been shown to begin when a germ line stem cell divides asymmetrically, generating one daughter cell that retains stem cell fate and a second daughter cell that adopts spermatogonia fate.

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RECOMMENDED RESOURCES

DEVELOPMENTAL BIOLOGY

Gilbert S: *Developmental biology*, ed 8, Sunderland, MA, 2006, Sinauer Associates, Inc.
Companion Web site: http://www.devbio.com/
Additional Web site: http://zygote.swarthmore.edu/
Slack JMW *Essential developmental biology*, ed 2, Malden MA, and Oxford, UK, 2006, Blackwell Publishing, Ltd.

MOUSE BIOLOGY

http://www.med.unc.edu/embryo_images/
http://genex.hgu.mrc.ac.uk/
http://mouseatlas.caltech.edu/index_content.html

HUMAN BIOLOGY

http://www.visembryo.com/baby/index.html

XENOPUS BIOLOGY

http://worms.zoology.wisc.edu/frogs/mainmenu.html
http://www-cbd.ups-tlse.fr/organismes/nieuwkoop/nieuwkoop.html

STEM CELL BIOLOGY

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http://www.cellmigration.org/index.shtml
http://embryology.med.unsw.edu.au/Movies/genital/germcell.htm

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9

PATTERNING THE ANTERIOR–POSTERIOR AXIS DURING *DROSOPHILA* EMBRYOGENESIS

KRISTY L. KENYON

Departments of Biology, Hobart and William Smith Colleges, Geneva, NY

INTRODUCTION

Drosophila melanogaster is a popular animal model for studying the genetic regulation of development. Remarkable progress has been achieved during the last several decades by using this invertebrate to investigate the molecular and cellular processes that control cell fate specification and determination. In particular, scientists have gained significant insight into the complex genetic mechanisms that create polarity within the fly egg and subsequently pattern the zygote along the various axes. This chapter provides a brief introduction into the molecular mechanisms that control the specification of the anterior–posterior (AP) axis during fly embryogenesis.

Fly development has proven to be an excellent model system for elucidating the complex molecular and cellular mechanisms involved in the acquisition of cell fates. Embryonic cells can regulate both temporal and spatial control of gene expression at the level of transcription and translation. As will be discussed in the following sections, the posttranscriptional modification of messenger RNA (mRNA) transcripts leads to a diverse array of cellular responses that range from the sequestering of molecules to specific locations to the targeted repression of mRNA translation. Morphogen gradients and signaling cascades create patterning within the embryo by generating differential responses within specific domains. Dynamic interactions among the various networks of transcription factors are instrumental in transforming the broad polarity of the early embryo into the segmented body plan of the fly larva and adult.

A newly fertilized *Drosophila* egg transitions through embryonic, larval, and pupal stages until it hatches into its adult form. For the first 12 cell cycles of embryogenesis, fly eggs cleave in a superficial pattern, with nuclear division occurring in the absence of cytokinesis. The fly embryo is considered

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a syncytial blastoderm during this stage of development, because the zygotic nuclei exist within a common cytoplasm (Figure 9.1). Mitosis initially happens within the central portion of the fertilized egg until the eighth nuclear division; during the subsequent cell cycles, nuclei migrate to the periphery. During the ninth division, a subset of nuclei migrates to the posterior region of the embryo; they will eventually become the pole cells, which are the future gametes of the organism. The process of cellurization begins after the thirteenth cell cycle. At the beginning of the cellular blastoderm stage (the fourteenth cell cycle), the embryo contains approximately 5000 nuclei that will become incorporated into a single layer of epithelium (not including the future gametes). Shortly thereafter, the movements of gastrulation begin to transform the embryo into a multilayered arrangement. The first cells to migrate inward do so along the ventral midline, and they will ultimately form a ventral tube of mesodermal cells internally. Subsequent invaginations at the anterior and posterior ends of the embryo generate endodermal gut structures. The future

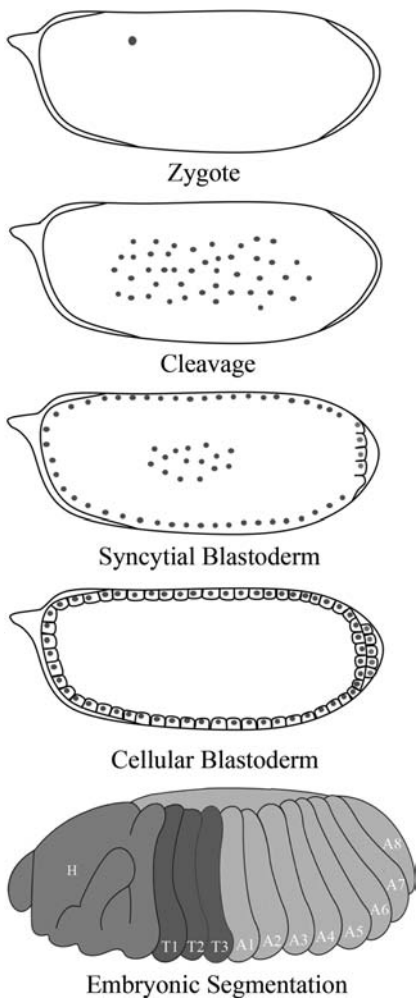

FIGURE 9.1 Embryonic stages leading to the formation of a segmented body plan. *Black dots* represent zygotic nuclei. *Dark gray dots*/cells represents those that will become the future pole cells (gametes). *H,* Head; *T,* thoracic; *A,* abdominal.

nervous system of the fly derives from ectodermal cells that migrate inward on the ventral side to form a layer of neuroblasts (neural precursor cells) between the mesoderm and the outer ectoderm.

During the later stages of gastrulation, the fly embryo undergoes a series of movements known as *germ band extension*. The ectodermal and mesodermal cells that line the ventral midline, which will generate the main trunk region of the embryo, are collectively referred to as the *germ band*. The germ band undergoes cellular movements that cause it to extend toward the posterior end of the embryo. At its maximum, the germ band wraps around to the future dorsal side of the embryo for a period of time. In this position, the first signs of segmentation begin to emerge as repeating external grooves along the germ band. From its most extended state, the germ band eventually retracts and continues the process of segmentation. After retraction has occurred, the appearance of distinct segments (three thoracic and eight abdominal) and an unsegmented head region become clearly visible (see Figure 9.1). The overall body plan of the embryo will be maintained as it transitions through subsequent life stages.

Specification of the AP axis begins before fertilization. During oogenesis, the oocyte becomes polarized by rearrangements within its cytoskeletal matrix and the localization of maternal mRNAs to specific poles. After fertilization occurs, the products of the maternal genes regulate zygotic genes that pattern the developing embryo into broad domains along the AP axis. The subsequent activation of gene networks further partitions the embryo into 14 repeating units known as *parasegments*. Parasegments are developmental compartments within the embryo; zygotic gene expression patterns reveal the sequential nature of these repeating parts. Parasegmental patterns of gene expression dictate the formation and conversion of the body plan into the segmented arrangement of the larval and adult stages.

I. MATERNAL CONTROL OF AXIS FORMATION

A. Establishing Polarity During Oogenesis

The generation of a basic body plan begins with critical events that generate polarity within the developing oocyte. In the ovary of a female fly, a single germ cell undergoes a series of cell divisions that generates 16 cells that are interconnected via cytoplasmic "ring" canals. Of these cells, one will develop into the oocyte precursor, whereas the remaining cells will become the supporting nurse cells. Somatic follicle cells surround the developing oocyte and nurse cells; the germ-line derived cells and the somatic follicle cells comprise the egg chamber. During oogenesis, nurse cells synthesize numerous mRNAs and proteins that will ultimately be transported into the oocyte precursor via ring canals. The follicle cells perform numerous tasks in generating the outer layers of the oocyte and participate in cell–cell interactions that are critical for cell fate specification (see Chapter 11).

Cellular events within the egg chamber lead to the creation of polarity within the oocyte well before fertilization occurs. Approximately mid oogenesis, the oocyte comes to reside in the posterior portion of the egg chamber; its nucleus is also located in a posterior position at this stage (Spradling, 1993; Gonzalez-Reyes and St. Johnston, 1995). This physical arrangement defines

the parameters of a molecular interaction that creates polarity in the surrounding follicle cells as well as in the oocyte. Nurse cells synthesize mRNA coding for a signaling molecule known as *gurken* (a member of the conserved epidermal growth factor superfamily) and export it to the oocyte (Neuman-Silberberg and Schüpbach, 1993; Saunders and Cohen, 1999; Cáceres and Nilson, 2005). Subsequently, *gurken* mRNA becomes preferentially localized to the cytoplasmic region between the oocyte nucleus and the plasma membrane (Cáceres and Nilson, 2005). During this stage of oogenesis, *gurken* mRNA is translated, and Gurken protein is released from the oocyte. Those follicle cells closest to the released Gurken respond via the *Drosophila* epidermal growth factor receptor protein known as Torpedo (Price et al., 1989; Neumann-Silberberg and Schüpbach, 1993); this event causes the responding cells to adopt a "posterior" fate (Gonzalez-Reyes et al., 1995; Roth et al., 1995). In turn, these specialized border cells initiate another signaling event—albeit through an unknown mechanism—that leads to an internal rearrangement of the cytoskeletal structure of the developing oocyte. The microtubule array that provides the basic framework for the oocyte becomes oriented such that the growing ends(+) (plus ends)– of the microtubules are located in the posterior end, whereas the minus ends are found in the future anterior end of the oocyte (reviewed in Steinhauer and Kalderon, 2006). Subsequent to this reorganization, the oocyte nucleus migrates along the microtubule array from its posterior position to an anterior–dorsal position. The nucleus remains in this position at the time of fertilization, and subsequent molecular events set up the future axes of the embryo (see Chapter 11).

The polarity of the cytoskeletal matrix plays an integral role in the localization of specific mRNAs within the oocyte. The sequestering of *bicoid* mRNA in the anterior region of the oocyte is required for the proper specification of anterior cell fates. *Bicoid* mRNA is transcribed in nurse cells and specific regions within its 3′ UTR mediate interactions with the proteins Exuperantia, Exuperantia-like, Swallow, and Staufen (Berleth et al., 1988; Ferrandon et al., 1997; Macdonald and Kerr, 1998; Schnorrer et al., 2000; Reichmann and Ephrussi, 2005). These proteins are involved in the transport of *bicoid* mRNA into the oocyte along the microtubule array (Cha et al., 2001; Arn et al., 2003). As part of a complex of RNA binding proteins, *bicoid* mRNA becomes attached to the microtubule organizing center (minus end) via dynein motor proteins (Schnorrer et al., 2000; Steinhauer and Kalderon, 2006). In this way, *bicoid* mRNA remains sequestered in the anterior region throughout fertilization and early cleavage stages.

At the posterior pole of the oocyte, another set of maternal factors become localized and function in the specification of posterior cell fates. Two central players in this process are *oskar* and *nanos* (Lehmann and Nüsslein-Volhard, 1986; Gavis and Lehmann, 1992; 1994). The localization and translational regulation of *nanos* involves a complex repertoire of maternal genes that include *oskar, staufen*, *vasa, valois,* and *tudor* (Lehmann and Nüsslein-Volhard, 1991; Gavis and Lehmann, 1992). Unlike the directed movement of other maternal genes, *nanos* mRNA appears to passively diffuse through the developing oocyte and become bound, via its 3′ UTR, to the translational inhibitor Smaug (Forrest et al., 2004; Nelson et al., 2004). In turn, Smaug protein recruits CUP, a protein that prevents the ability of *nanos* mRNA to become incorporated into the ribosomal machinery. The majority of *nanos* mRNA is prevented from being translated by this mechanism.

However, a subset of *nanos* mRNA does become specifically sequestered in the posterior pole of the oocyte. The prerequisite for this process is the prior localization of *oskar* mRNA and Staufen protein to the microfilaments found in the cortex of the posterior pole (Brendza et al., 2000; Hatchet and Ephrussi, 2004). The transport of *oskar* mRNA to the posterior pole requires the activity of the motor protein kinesin I, which moves *oskar* messages along microtubules in the plus-end direction (Brenzda et al., 2000, Cha et al., 2002). Staufen allows for the translation of the *oskar* transcript in the posterior pole; in turn, Oskar protein binds to specific regions within the 3′ UTR of *nanos* mRNA (Brendza et al., 2000; Hatchet and Ephrussi, 2004). If Oskar protein comes in contact with the complex of *nanos*-Smaug-CUP, Oskar can cause the disassociation of CUP from the complex, thereby allowing for *nanos* mRNA to be bound within a complex of RNA binding proteins in this region (Forrest et al., 2004; Nelson et al., 2004).

The maternal genes that become localized to the posterior pole of the oocyte have important roles for two key aspects of embryogenesis: the specification of abdominal cell fates and the formation of the germ line. As mentioned previously, the pole cells (future gametes) arise from the posterior pole of the early embryo; their development is controlled by germ plasm determinants that exist in this region. Although this chapter will not address this important topic, it is worth noting that Oskar, Vasa, and Nanos are essential members of the germ plasm and that their role in germ-cell formation appears to be conserved among different species (reviewed in Saffman and Lasko, 1999).

B. "Maternal Effect" Genes Pattern the Early Embryo Along the Anterior–Posterior Axis

Three systems of maternally derived genes operate to pattern the fertilized egg as it develops into a syncytial blastoderm. The research of Sander and colleagues (1975) provided the first experimental evidence that gradients are involved in the establishment of polarity within the developing insect egg. Subsequently, Nüsslein-Volhard, Wieschaus, and their colleagues successfully identified numerous genes involved in axis formation by their systematic screening of mutant phenotypes at the genomic level (reviewed in Ephrussi and St. Johnston, 2004). On the basis of their work and that of others, we now know a great deal about the genetics of early patterning events during fly embryogenesis. The initial genes critical to this process are often collectively referred to as "maternal effect" genes based on the fact that they are transcriptionally derived from the maternal genome. Of this group, the genes *bicoid* and *nanos* play critical roles in creating the initial polarity along the AP axis. The designation of "anterior" is driven by the activities of Bicoid, whereas "posterior" specification relies on the activity of Nanos. In addition to these two systems, the specification of the termini of the embryo (acron, telson) makes use of a signaling cascade involving the Torso receptor protein.

The localization of *bicoid* and *nanos* mRNA within the oocyte at the time of fertilization has key implications for guiding the development of the early embryo. At the time of fertilization, molecular events allow for the translation of these sequestered mRNAs. Whereas the mRNAs are restricted, Bicoid and Nanos proteins can freely diffuse within the cleavage stage embryo. Bicoid and Nanos proteins regulate the activity of other maternal genes, including *hunchback* and *caudal,* respectively. Unlike *bicoid* and *nanos* mRNA, maternal *hunchback* and *caudal* mRNA are distributed uniformly throughout the

egg cytoplasm (described later). The interactions among these maternal factors ultimately create gradients within the embryo that will drive gene-expression patterns during later stages of development (Figure 9.2).

Shortly after fertilization, the sequestered *bicoid* mRNA becomes translated, and a gradient of Bicoid protein forms during the subsequent cell cycles. The expression of Bicoid is highest in the anterior portion of the embryo, and it gradually tapers off posteriorly (Driever and Nüsslein-Volhard, 1988a, 1988b). Loss-of-function analyses indicate the importance of a Bicoid gradient in patterning the anterior portion of the fly embryo. Embryos lacking Bicoid protein lack anterior head structures (acron, head, thorax); bicoid^{-} mutants reveal a body plan that has a telson–abdomen–telson arrangement (Frohnhöfer and Nüsslein-Volhard, 1986). Defects in those genes responsible for bicoid localization (e.g., *exp*, *swallow*) reveal specific defects in axial patterning that are consistent with alterations in a Bicoid protein gradient (Berleth et al., 1988; Driever and Nüsslein-Volhard, 1988a; St. Johnston et al., 1989). Proper axis formation can be restored in *bicoid* mutants by the exogenous addition of *bicoid* mRNA at the anterior region (Driever and Nüsslein-Volhard, 1990). Also, the misexpression of *bicoid* mRNA is sufficient to induce anterior development in any part of the embryo, and this can lead to the creation of embryos with "heads" at both poles (Driever and Nüsslein-Volhard, 1990). Altering the levels of Bicoid gradient leads to concentration-dependent changes in zygotic gene expression, as will be discussed later in this chapter.

Bicoid is a member of a conserved family of transcriptional regulators that all contain a homeodomain DNA-binding motif (Berleth et al., 1988). Bicoid performs numerous functions in the specification of anterior cell fates. One key function of this protein is to regulate the maternal gene *caudal*. Caudal is a transcription factor that regulates genes involved in posterior cell fate determination (i.e., abdomen). As a maternal gene, *caudal* mRNA is

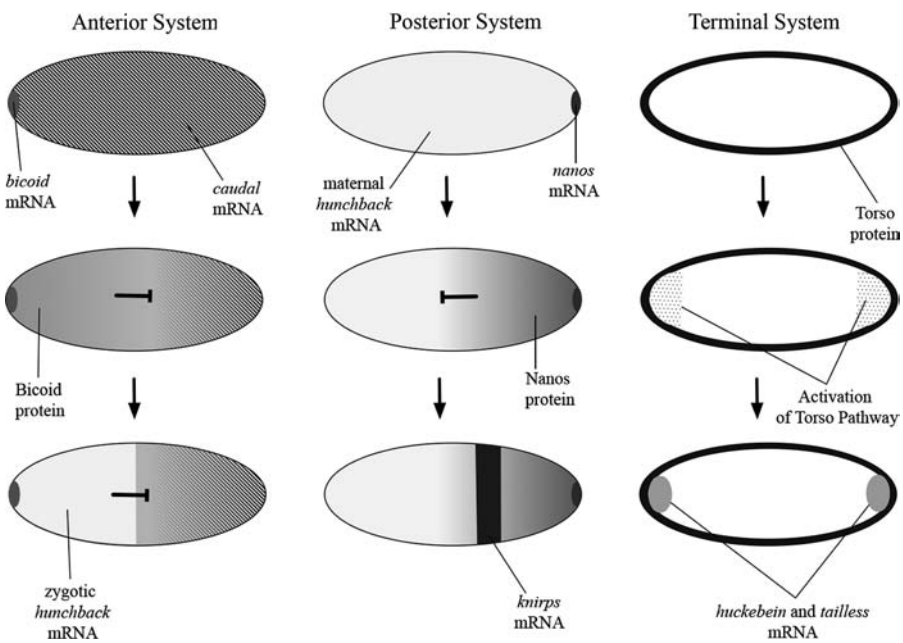

FIGURE 9.2 Schematic representation of the three maternal gene networks that establish polarity along the AP axis of the fly embryo. (See color insert.)

synthesized in the nurse cells and transported to the oocyte, where *caudal* transcripts can be found throughout the fly egg. Despite its ubiquitous mRNA expression, Caudal protein is expressed in a posterior-to-anterior gradient within the early embryo (MacDonald and Struhl, 1986). If expressed in the anterior region of the embryo, Caudal protein can alter the development of the head and thorax (Mlodzik et al., 1990). Thus, one aspect of "anterior" specification must involve the negative regulation of *caudal* mRNA. To this end, Bicoid acts as an inhibitor by binding to the 3′ UTR of *caudal* mRNA, thereby blocking its ability to be translated (see Figure 9.2; Chan and Struhl, 1997; Rivera-Pomar et al., 1996; Niessing et al., 2000). Bicoid also plays a critical role in regulating the transcription of several zygotic genes. Bicoid has a unique distinction among regulatory proteins, because it can bind to both DNA and RNA via the homeodomain (Baird-Titus et al., 2005). As a transcription factor, Bicoid activates the zygotic expression of *hunchback* (zygotic) as well as of other genes (*buttonhead, orthodenticle, empty spiracles*) required for the anterior patterning of the head and thorax (Driever and Nüsslein-Volhard, 1989; Struhl et al., 1989; Cohen and Jürgens, 1990; Finkelstein and Perrimon, 1990; Grossniklaus et al., 1994; Gao and Finkelstein, 1998). The Bicoid-dependent regulation of *hunchback* is particularly critical for establishing a Hunchback protein gradient in the anterior portion of the embryo (see Figure 9.2). Bicoid and Hunchback work in a cooperative manner to regulate a number of target genes required in for the development of the head and thorax regions (described below).

In the future posterior region of the embryo, Nanos protein plays a pivotal role in creating a different set of gradients (Wang and Lehmann, 1991). Embryos lacking Nanos are missing the abdominal segments (acron–head–thorax–telson) (Lehmann and Nüsslein-Volhard, 1986; Schüpbach and Wieschaus, 1986). Although *nanos* mRNA does exist throughout the cytoplasm of the early embryo, it can only be translated in the posterior pole in accordance with the context-dependent actions of other proteins such as Oskar (reviewed in Wilhelm and Smibert, 2005). After translation has occurred, Nanos protein diffuses from the posterior pole and creates an opposing gradient to that of Bicoid (see Figure 9.2). The function of Nanos in the posterior region is to block the translation of *hunchback* mRNA by associating with the RNA-binding protein Pumilio (Barker et al., 1992; Murata and Wharton, 1995). In the posterior portion of the embryo, the Pumilio protein recruits Nanos in a binding interaction involving the Nanos response element (NRE) located in the 3′ UTR region of *hunchback* mRNA (Wrenden et al., 1997; Sonoda and Wharton, 1999). The binding of the Nanos–Pumilio complex leads the deadenylation of *hunchback* mRNA (Wrenden et al., 1997); this complex may also inhibit translation in other ways not involving the poly A tail (Chagnovich and Lehmann, 2001).

Thus, two sets of opposing gradients establish polarity along the AP axis in the embryo. Bicoid and Hunchback exist in anterior-to-posterior gradients within the syncytium. These two transcription factors directly influence the zygotic gene expression necessary for head and thorax development. Conversely, Nanos and Caudal proteins are expressed in a posterior-to-anterior gradient. In the absence of Bicoid and Hunchback, Caudal regulates the transcription of the zygotic genes required for abdominal cell fate specification in the posterior portion of the embryo (see Figure 9.2).

A third set of maternal genes is required to specify the terminal regions of the fly embryo, the acron (the unsegmented anterior portion of the head), and the telson. Whereas morphogen gradients within the egg control the development of the head, thorax, and abdomen, the development of the acron and telson involve specific interactions between the oocyte and somatic follicle cells. The receptor tyrosine kinase *torso* plays a critical role in this process (Klingler et al., 1988; Sprenger et al., 1989). *Torso* mRNA is transcribed in the ovarian nurse cells and subsequently transported to the developing oocyte. Upon fertilization, the *torso* mRNA is translated, and the receptor protein is inserted along the entire plasma membrane of the embryo (Casanova and Struhl, 1989). Interestingly, it is the activation of the receptor that is spatially restricted to the terminal ends of the fertilized egg (see Figure 9.2). During normal development, the signal for Torso is active only at the two poles of the embryo. Gain-of-function mutants for Torso exhibit a body plan that exhibits only an acron and an enlarged telson; these mutant embryos lack head, thorax, and abdominal segments (Klingler et al., 1988). The putative ligand for the Torso receptor is the secreted protein Trunk. Trunk requires proteolytic processing to generate a C-terminal fragment that can activate the Torso receptor (Casanova et al., 1995; Casali and Casanova, 2001). The modification of the Trunk protein must be spatially localized, because *trunk* mRNA—similar to its receptor—is also expressed throughout the oocyte. *Torso-like,* which is a gene that is expressed in the anterior and posterior border follicle cells, appears to be a key mediator of Trunk processing and the restricted activation of Torso at the two poles of the embryo (Savant-Bhonsale et al., 1993; Martin et al., 1994; Stevens et al., 2003).

After the Torso receptor has become activated, it elicits a signaling cascade at both ends of the embryo that relieves the inhibited expression of the zygotic genes *tailless* and *huckebein*. These two transcription factors are required for the specification of both the acron and the telson. In response to the Torso signaling pathway, the transcriptional repressor Groucho becomes inactivated; the removal of Groucho repression at the terminal poles allows for the zygotic expression of *tailless* and *huckebein* in these regions (Paroush et al., 1997). Considering that the anterior and posterior termini express the same genes, how do embryonic nuclei distinguish between an acron or a telson fate? The answer lies in the presence of the morphogen gradient of Bicoid that overlaps with the terminal system in the anterior part of the embryo. The cellular conditions created by the combined activity of the Bicoid and Torso systems leads to the specification of an acron in this region (Pignoni et al., 1992). In fact, recent work by Schaeffer and colleagues (2000) has shown that high levels of Bicoid can rescue patterning defects caused by the loss of Torso signaling in the anterior region of the embryo. This suggests that the Torso and Bicoid systems have redundant functions in specifying the anterior terminal portion of the fly embryo (Schaeffer et al., 2000).

II. SEGMENTATION OF THE FLY EMBRYO

A hierarchy of zygotic gene expression controls the transitions of the early fly embryo from the syncytial blastoderm stage to the segmented body plan revealed during subsequent life stages. The different cytoplasmic environments created by the numerous maternal gene products discussed previously

contribute to the zygotic expression of gap genes in overlapping domains along the AP axis. As transcription factors, the products of these genes in turn regulate the expression of the pair-rule genes, which have expression domains that reveal a parasegmental organization within the embryo. The pair-rule gene products in turn regulate the expression of the segment polarity genes. Segment polarity genes work to maintain the parasegmental patterning via cell–cell signaling events that dictate the cell fate decisions that define the boundaries of the parasegments. The final step in the segmentation process is the acquisition of segment identity; the combined activity of the pair-rule genes and gap genes regulate the expression of the homeotic selector genes, which are responsible for the specification of individual segmental fates.

A. Dynamic Expression of Gap Genes and Their Protein Products Define Broad Domains within the Embryo

The initial identification of the gap genes began with the observations of mutant embryos displaying large "gaps" that corresponded with the specific loss of several contiguous segments; polarity within the embryo itself was not affected (Nüsslein-Volhard and Wieschaus, 1980). Subsequent research led to the characterization of other mutants displaying similar phenotypic patterns in which broad domains of the embryonic body plan were absent. The major members of this group include *hunchback* (the zygotic form), *Krüppel, knirps, giant, huckebein, tailless, orthodenticle,* and *buttonhead* (Table 9.1). All known gap genes code for transcription factors, and their protein products further establish the precise domains of overlapping gene expression within the

TABLE 9.1 Major *Drosophila* Genes Involved AP Specification and Their Vertebrate Homologs

Category	Gene name	Cellular function	Vertebrate‡
Maternal	*gurken*	Secreted growth factor (EGF family)	EGF
	torpedo	Tyrosine kinase receptor (EGF family)	EGFR
	oskar	Novel RNA binding protein	—
	bicoid	Homeodomain transcription factor	Hox3
	caudal	Homeodomain transcription factor	Xcad(s)/Cdx(s)
	hunchback	Zinc finger transcription factor	Ikaros
	nanos	RNA binding protein	Xcat-2
	torso	Tyrosine kinase receptor	—
	torso-like	Novel protein, putative activator of Trunk	—
	trunk	Putative ligand for Torso	—
Zygotic Gap	*hunchback*	Zinc finger transcription factor	Ikaros
	Krüppel	Zinc finger transcription factor	—
	giant	Basic leucine zipper transcription factor	—
	knirps	Transcription factor/steroid recepto	—
	tailless	Transcription factor/steroid receptor	TLXs
	huckebein	Zinc finger transcription factor	—
	orthodenticle	Homeodomain transcription factor	Otx1/2
	buttonhead	Zinc finger transcription factor	mBtd /Sp1-related
	empty spiracles	Homeodomain transcription factor	Emx1/2

continued

TABLE 9.1 Major *Drosophila* Genes Involved AP Specification and Their Vertebrate Homologs—Cont'd

Category	Gene name	Cellular function	Vertebrate[‡]
Pair-rule			
Primary	*even-skipped*	Homeodomain transcription factor	Evx
	hairy	Basic helix-loop-helix transcription factor	Multiple Hes
	runt	Novel transcription factor	AML1/PEBP2
Secondary	*fushi tarazu*[*]	Homeodomain transcription factor	—
	odd-paired	Zinc finger transcription factor	Zic1–3/GL1
	sloppy-paired	Forkhead transcription factor	BF1/HBF2
	odd-skipped	Zinc finger transcription factor	Osr1/2
	paired	Paired, homeodomain transcription factor	Pax3
Segment polarity	*engrailed*	Homeodomain transcription factor	En1, 2
	wingless	Signaling ligand	Wnt1- Wnt12
	cubitus interruptus	Zinc finger transcription finger	Gli-3
	hedgehog	N-terminus signaling ligand	Sonic, Indian, desert, banded hedgehog–
	fused	Serine-threonine kinase	—
	dishelvelled	Cytoplasmic transducer of *wg* signaling	Dsh/Dvl
	zeste white3[†]	Serine/threonine kinase	GSK-3
	armadillo	Transcriptional regulator	β-catenin
	patched	Transmembrane receptor for hedgehog	Patched
	frizzled	Transmembrane receptor for wingless	Many Frizzleds
	gooseberry	Paired homeodomain transcription factor	Pax3–7
	pangolin	HMG transcription factor	Lef/TCF

*Controversial as to whether this gene is a primary or secondary.
[†]Also known as *shaggy*.
[‡]Partial list of vertebrate homologs obtained from http://flybase.bio.indiana.edu/allied-data/lk/interactive-fly/aimain/1aahome.htm.

embryo. The overall profile of gap gene expression creates different developmental regions along the AP axis.

Gap gene expression marks the transition from maternal to zygotic control of gene regulation. As transcription factors, Bicoid, Hunchback, and Caudal act in a concerted manner to regulate gap gene expression along the AP axis. In particular, the Bicoid gradient provides important positional information to nuclei within the syncytium that leads to an amplification of zygotic *hunchback* gene expression in the anterior portion of the embryo. The concentration of Bicoid protein directly affects zygotic *hunchback* transcription (Driever and Nüsslein-Volhard, 1988a, 1988b). Using gain-of-function analyses, researchers demonstrated that increasing Bicoid concentration caused the zygotic *hunchback* expression domain to extend more posteriorly (Driever and Nüsslein-Volhard, 1988b; Struhl et al., 1989). In fact, a twofold change in Bicoid protein is sufficient to switch the expression of *hunchback* from "off" to "on" (Struhl et al., 1989).

The threshold response of *hunchback* gene expression to a Bicoid concentration gradient was pivotal in supporting the view that the early patterning of the fly embryo is driven by the activity of morphogen gradients, as proposed by Wolpert (1969). More recently, researchers have begun to look more closely at the relationship between the Bicoid gradient and the precision of the gene-expression patterns of its targets, such as *hunchback* mRNA. Gap gene expression is characterized by the definitive nature of these domains, with tight patterning of the borders. In light of this model, many questions arise regarding the parameters that could influence the creation of the Bicoid gradient and its effectiveness in providing both spatial and proportional information for the developing embryo. Internal factors such as mRNA concentration, translation rates of *bicoid* messages, degradation of Bicoid protein, and environmental conditions (e.g., temperature) have significant implications for the generation of a morphogen gradient. In fact, research by Houchmandzadeh and colleagues (2002) has shown that the Bicoid gradient is highly variable from embryo to embryo according to protein expression profiles during the fourteenth cell cycle (i.e., the beginning of cellularization). This variability in the Bicoid gradient does not affect the precision of *hunchback* mRNA or protein expression based on the relative position of the posterior border of its expression domains. The authors suggest that there must be other mechanisms in place that allow for the "filtering" of the Bicoid gradient at the level of *hunchback* expression and that the RNA-binding protein Staufen may act in this capacity. However, as suggested in a recent review by Yucel and Small (2006), the idea of "filtering" must be evaluated more closely to consider temporal and spatial changes in gene transcription and translation that may account for these apparent contradictions to the model of a threshold-dependent activation by the Bicoid concentration gradient. Moreover, other mechanisms, such as cooperative DNA binding and combinatorial control of transcription, have also been shown to affect the Bicoid-dependent patterning in the anterior region (Simpson-Brose et al., 1994; Ma et al., 1996; Lebrecht et al., 2005). The role of Bicoid in patterning the anterior region of the embryo remains an active area of investigation (Ephrussi and St. Johnston, 2004).

The establishment of a Hunchback gradient clearly affects the initiation of other gap gene expression along the AP axis. Varying levels of Hunchback and Bicoid dictate complex patterns of transcriptional activation and repression in a context-dependent manner. For example, the gap genes *buttonhead*, *empty spiracles*, and *orthodenticle* all required high levels of Bicoid and the synergistic activity of Hunchback for proper activation (Simpson-Brose et al., 1994; Reinitz et al., 1995; Gao and Finkelstein, 1998). The expression of *Krüppel* in a single broad domain in central region of the embryo also depends on the Bicoid and Hunchback gradients. *Krüppel* gene expression responds to threshold levels of Bicoid (high) and Hunchback (low); high levels of Hunchback repress the expression of *Krüppel* in the most anterior region of the embryo (Struhl et al., 1992). The borders of this domain are further defined by the repressive activity of Giant (Wu et al., 1998). Giant expression becomes centralized into two bands of expression: one in the anterior and one in the posterior (see Figure 9.2). The anterior expression of *giant* depends on Bicoid and Hunchback, whereas the posterior band of expression depends on the Caudal gradient in the posterior region of the embryo (Schulz and Tautz, 1995; Rivera-Pomar et al., 1996).

The morphogenic activities of Bicoid, Hunchback, and Caudal initiate gap gene expression that regionalizes the embryo along the AP axis. These subdomains are further defined and maintained by complex interactions among the various gap gene products. Repressive interactions among these transcriptional regulators play an essential role in defining the borders of gap gene expression. For example, Giant and Krüppel act to repress the other's expression, and a similar relationship exists between Hunchback and Knirps (Kraut and Levine, 1991; Clyde et al., 2003). Computational analyses have been used to dissect the intricate processes necessary for the initiation and stabilization of gap gene expression. Using quantitative gene-expression data sets, Jaeger and colleagues (2004) developed a "gene circuit" model to investigate the regulatory relationships among gap genes between the thirteenth and fourteenth cell cycles. Their modeling data support the idea that mutually repressive interactions (Hunchback/Knirps and Giant/Krüppel) are critical to stabilizing gap gene expression patterns along the AP axis. Furthermore, their work provides evidence that unidirectional repressive interactions have a significant role in defining the anterior and posterior borders of gap gene expression (Figure 9.3; Jaegar et al., 2004; Monk, 2004). Although limited to a specific stage of development, the work of Jaegar and colleagues demonstrates the power of mathematical modeling for deciphering the complex interactions that govern gap gene expression.

Regionalization within the syncytial blastoderm is thus created by overlapping patterns of gap mRNA expression. The major known gap genes all encode transcription factors that can either activate or repress gene expression in a context-dependent manner. In conjunction with maternal factors, gap proteins regulate the further partitioning of the embryo into discrete developmental units.

B. Pair-Rule Gene Expression Reveals the Parasegmental Organization of the Embryonic Body Plan

Periodicity within the embryonic body plan emerges just before the process of cellularization. A key hallmark of this transition is the expression of the pair-rule genes in a distinctive striped pattern along the AP axis (Figure 9.4). The expression of this class of genes marks the first indication of the segmentation process, although the stripes do not correspond specifically with the future segments. The expression patterns of the pair-rule genes demarcate the formation of parasegments, which are developmental compartments within the early embryo. Each parasegment represents a population of cells that will develop as a unit to generate the individual segments (Martinez-Arias and Lawrence, 1985). Parasegmental organization is out of phase with the segments. Thus, each segment arises from the posterior domain of one parasegment and the anterior domain of the adjacent parasegment (Figure 9.5).

Pair-rule genes are expressed in a "zebra" striped pattern with bands of expressing cells alternating with bands of nonexpressing cells. For example, the pair-rule gene *even-skipped* is expressed in the odd parasegments but not the even parasegments (see Figure 9.4). The pair-rule genes are divided into primary and secondary categories (see Table 9.1). Primary pair-rule genes include *even-skipped, hairy,* and *runt*. The expression of the primary pair-rule genes is dictated by the actions of gap and maternal proteins. Each stripe of expression is controlled by interactions between these transcriptional

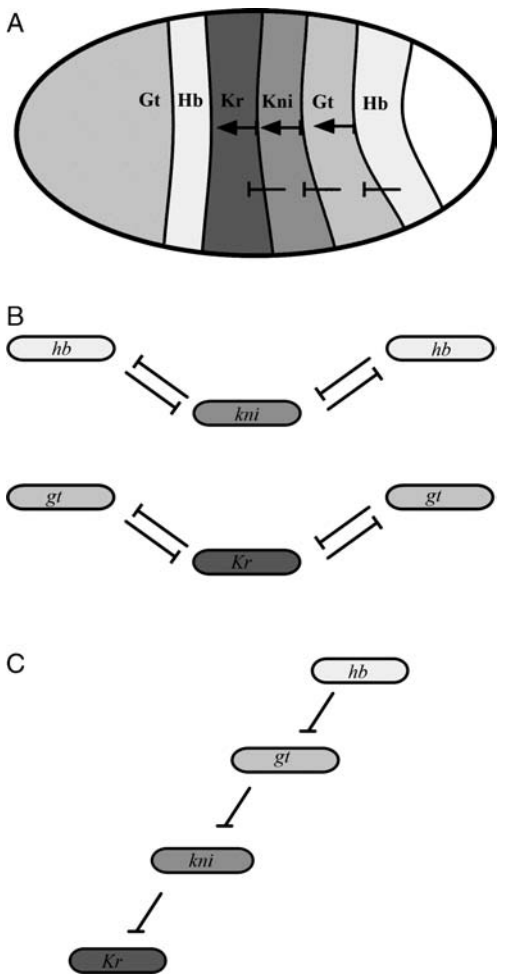

FIGURE 9.3 **The expression domains and regulatory interactions controlling gap gene expression in the syncytial blastoderm. A,** Schematic representation of a stage 14 embryo revealing gap expression domains (overlaps in gene expression are not shown). Gap genes expressed in the posterior region repress the expression of their anterior neighbors (bars). The arrows indicate the anterior shift in the posterior borders of *gt*, *kni*, and *Kr* expression. **B,** Mutual repressive interactions among specific gap proteins establish the basic pattern at this stage. **C,** The asymmetric repression of anterior genes by their posterior neighbors leads to an anterior shift in gap expression domains. *Gt,* Giant; *Hb,* hunchback; *Kni,* knirps; *Kr,* Krüppel. (Adapted with permission from Monk, 2006.)

regulators and specific enhancer regions in the DNA. Enhancers act as molecular switches by responding to the varying cellular conditions along the AP axis.

The transcriptional regulation of the *even-skipped* gene is the best known example of how enhancer elements act in a modular manner to create the unique striped pattern of pair-rule expression. The expression of *even-skipped* is initially detected at a low level in most of the nuclei of the syncytial blastoderm at cell division 12; soon thereafter, its expression becomes refined into

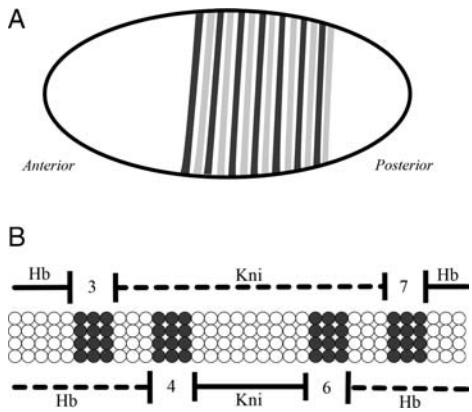

FIGURE 9.4 **A,** Schematic representation of the alternating expression patterns of *even-skipped* (*black bars*) and *fushi tarazu* (*gray bars*) in the syncytial blastoderm. The expression of these two pair-rule genes underscores the parasegmental organization of the embryo. **B,** Mutually repressive interactions between Hunchback (Hb) and Knirps (Kni) regulate the domains of their gradients. In zygotic nuclei of the embryo, specific enhancers of the *eve* gene differentially respond to these repressive signals (either Hb or Kni), which leads to the precise locations of stripes 3, 4, 6, and 7 expression (black). (Adapted with permission from Clyde et al., 2003.)

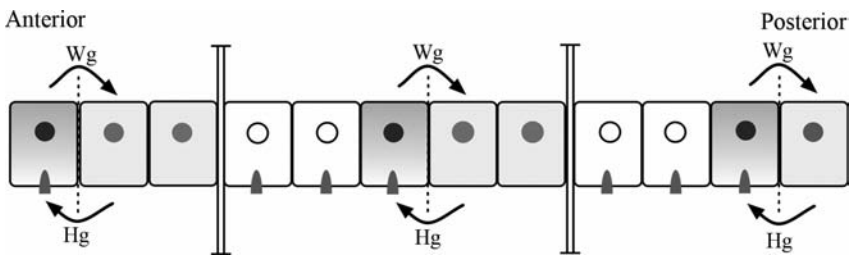

FIGURE 9.5 Schematic representation of the compartmental boundaries established by Wingless (blue) and Engrailed/Hedgehog (pink) signaling pathways. The dotted line indicates the parasegmental boundary; the vertical bar indicates the segmental boundary. Patched receptors (red crescent) are asymmetrically distributed within each segment contributing to the differential responses to Hedgehog signaling. (See color insert.)

the characteristic pattern of seven transverse stripes (Frasch et al., 1987). Each stripe of expression is created by the differential response of specific enhancer elements to different concentrations of gap and maternal proteins. For example, stripe 2 expression is dependent on an enhancer element (~500 base pairs in length) that is upstream of the transcriptional start site of the *even-skipped* gene (Small et al., 1992; Stanojevíc et al., 1991). This enhancer region contains five binding sites for Bicoid, one for Hunchback, three for Giant, and three for Krüppel. The binding sites of these activators and repressors are in relatively close proximity, which suggests the potential for competitive binding interactions. Transcriptional output appears to depend on the differential response of the enhancer element to the changing concentrations of both activators and repressors (see Figure 9.4). At threshold concentrations, Bicoid and Hunchback act in a cooperative manner to activate transcription when bound to this enhancer. Both Krüppel and Giant are repressors of *even-skipped*

expression in stripe 2. Giant repression contributes to the molecular interactions that limit the anterior border of stripe 2, whereas Krüppel repression helps to position the posterior border (Small et al., 1992). Giant and Krüppel appear to act in a similar manner in the defining the borders of *even-skipped* stripe 5 expression. In this context, Giant negatively regulates expression on the posterior border, whereas Krüppel repression occurs at the anterior border (Fujioka et al., 1999).

Repressive interactions involving Knirps and Hunchback dictate the formation of four other stripes of *even-skipped* expression (see Figure 9.4; Clyde et al., 2003). Two enhancers are involved in the expression of stripes 3+7 and stripes 4+6, respectively. Both enhancers respond to the environmental conditions established by the regulatory relationship between Hunchback and Knirps. These two transcription factors mutually repress each other (in addition to its anterior band of expression, Hunchback is also expressed in a posterior band at this stage). These interactions create a domain of Knirps expression flanked by two bands of Hunchback expression (see Figure 9.3). Knirps represses the expression of *even-skipped* in the region between stripes 4 and 6, thereby setting the internal borders of these stripes (posterior for 4, anterior for 6). The external borders of stripes 4+6 are defined by the repressive actions of Hunchback. The enhancer controlling stripes 3+7 respond in a similar manner to the Hunchback and Knirps gradients; in this context, the enhancer is able to "interpret" different concentration levels of the two factors as compared with the enhancer for stripes 4+6. The number and the affinity of binding sites dictate the differential responses of these two enhancers (Clyde et al., 2003).

The initiation of pair-rule gene expression is based on the complex interactions of the gap and maternal proteins with DNA regulatory elements. The products of the primary pair-rule genes work to both stabilize and maintain their parasegmental expression domains. All pair-rule genes encode transcription factors; thus, they function in further regulating gene expression within the embryo. Historically, pair-rule genes have been distinguished as either "primary" or "secondary," depending on the inputs that regulate their expression (see Table 9.1). Primary pair-rule genes require the activity of the gap and maternal proteins to define their expression domains. By contrast, secondary pair-rule genes make use of input from the protein products of the primary pair-rule genes to refine their expression patterns into the characteristic striped pattern. For example, *fushi tarazu* (*ftz*) is first expressed at a low level in a broad band that spans the future segmented part of the embryo (Karr and Kornberg, 1989). As with other pair-rule genes, its initial broad expression becomes stabilized into a pattern of seven stripes. Hairy acts to repress *ftz* expression, thus aiding in the creation of interstripe regions that no longer express *ftz* (Tsai and Gergen, 1995). Although repression contributes to creating interstripes, the increased activation of gene transcription also plays an important role in stabilizing the stripes of expression. For example, Fushi tarazu protein, which is a homeodomain transcription factor, autoregulates its own expression by interacting with a specific enhancer element within its own promoter sequence (Schier and Gehring, 1992). In addition to transcriptional regulation, other mechanisms, such as mRNA stability, have been implicated in creating the stripes of *ftz* expression (Riedl and Jacobs-Lorena, 1996).

The expression of the pair-rule genes defines the parasegmental organization of the embryo. Each set of nuclei within an individual parasegment expresses a specific constellation of pair-rule genes and proteins. As transcrip-

tion factors, pair-rule proteins play a direct role in the subsequent steps of segmentation. Direct targets of these transcription factors include the segment polarity genes and the homeotic selector genes.

C. Segment Polarity Genes Stabilize the Periodicity of Parasegments and Define the Anterior–Posterior Patterning of Individual Segments

Before the onset of gastrulation, the patterning of the embryo depends on the unique nature of molecular interactions that occur within the syncytium. After they have been translated within particular domains, maternal and zygotic transcription factors affect the transcriptional output of embryonic nuclei as their proteins diffuse within the syncytium. However, the mRNAs and proteins that generate the initial patterning along the AP axis are short lived, and the maintenance of these patterns must become stabilized as the embryo transitions into a cellular arrangement. By the time that the embryo consists of individual cells, molecules involved in cell–cell communication are required for reinforcing gene-expression patterns that will govern the formation of the future segments.

The partitioning of the embryo into parasegments is an essential step in the building of the fly body plan. The allocation of cells into non-overlapping cell populations organizes the embryo into a series of compartments (Martinez-Arias and Lawrence, 1985). In the context of development, cells comprising a compartment display two important characteristics: they do not mix with cells of adjacent compartments, and they develop in a lineage-restricted manner (Lawrence and Struhl, 1996). During the specification of the AP axis, cells of each parasegment become organized into an anterior compartment and a posterior compartment. The boundaries that exist between parasegmental compartments are an important source of patterning within the segments (Lawrence and Struhl, 1996; Dahmann and Basler, 1999).

At the gene level, compartmentalization depends on the actions of the segment polarity genes. Mutations in segment polarity genes generally result in polarity errors within individual segments, such as duplications, deletions, or reversals in segment orientation (Nüsslein-Volhard and Weischaus, 1980). The expression of the segment polarity genes defines parasegmental divisions within the embryo. For example, *engrailed (en),* a homeodomain transcription factor, is expressed in the anterior compartment of every parasegment; the cumulative pattern of *en* expression consists of 14 transverse stripes (Fojse et al., 1985). Complex interactions of pair-rule transcription factors control the restricted and repeating nature of the *en* expression pattern. In general, transcriptional repression has an equally important role to that of transcriptional activation in controlling segment polarity gene expression. Different sets of pair-rule genes regulate *en* expression within the even and odd parasegments. For cells within even-number parasegments, Fushi tarazu activates *en* expression, whereas Eve and Odd-paired act to confine its expression to only those cells in the anterior border of the parasegments (DiNardo and O'Farrell, 1987; Mullen and DiNardo, 1995). Even-skipped, Paired, and Sloppy-paired are required for the activation and refinement of *en* in the odd-number segments (Fujioka et al., 1995; Jaynes and Fujioka, 2004).

Engrailed plays a key role in determining regional identity within the parasegment as well as the future segment. Engrailed acts as a "selector" gene in the

process of segmentation. *Engrailed* continues to be expressed in the descendants of its original domain and thus acts as a molecular signal by informing cells about their position as part of the anterior compartment of the parasegment and later as part of the posterior compartment of the segment (Lawrence, 1992). Engrailed plays an integral role in defining AP compartmental boundaries as part of a regulatory loop involving the Wingless (of the Wnt gene family) and Hedgehog signal transduction pathways. The cell–cell signaling interactions that create and maintain this boundary set in motion a cascade of events that pattern cells according to their position from the boundary.

The expression of *en* and *wingless* within the parasegment initiates the formation of boundaries between parasegmental compartments. The pair-rule transcription factors restrict the the initial expression of *wingless* to those cells that lie in the posterior border of each parasegment (Baker, 1988; Fujioka et al., 1995; Mullen et al., 1995). This basic pattern—a stripe of *wingless*-expressing cells in one parasegment abutting a stripe of *en*-expressing in the next parasegment—is reiterated along the AP axis (see Figure 9.5). The maintenance of this pattern requires cell–cell interactions that ensure the continual expression of both *wingless* and *en*. In *wingless*-expressing cells, mRNA transcripts are translocated to the apical side of the cell, where the translation and secretion of Wingless protein occurs (Simmonds et al., 2001; Wilkie and Davis, 2001). The neighboring *en*-expressing cells bind Wingless (via Frizzled receptors), thereby causing the activation of the Wnt signal transduction pathway; ultimately, this pathway leads to the continued expression of *en* expression in these cells (Siegfried et al., 1994).

As a transcriptional regulator, Engrailed positively regulates its own expression and the expression of *hedgehog* mRNA in a cell-autonomous manner (Tabata et al., 1992; Mohler and Vani, 1992). Engrailed-expressing cells secrete Hedgehog; the reception of Hedgehog signaling occurs locally in cells expressing its receptor Patched. The binding of Hedgehog elicits a signal transduction pathway that ultimately leads to the maintenance of *wingless* expression in the adjacent cells. The activation of *wingless* expression by Hedgehog is restricted to the row of cells just anterior to those expressing Engrailed/Hedgehog (see Figure 9.5; reviewed in Hatini and DiNardo, 2001). Thus, a reciprocal loop of gene expression controlled by Wingless and Hedgehog is created within each parasegment, and the boundary between these two different cell types becomes stabilized.

The cells at each boundary act as a signaling center in further patterning of cell fate identity across each parasegment. After the initiation of the signaling center, the surrounding cells begin to respond asymmetrically to Wingless and Hedgehog based on complex interactions involving members of each transduction pathway. Controlling receptor expression, especially in the context of Hedgehog, appears to be one mechanism involved in this process. The asymmetric distribution of Wingless protein appears to be another means of achieving polarity within each parasegmental population (Hatini and DiNardo, 2001; Lander et al., 2002; Casal et al., 2002). Embryonic cells are guided toward different developmental pathways based on their interpretation of the Hedgehog and Wingless signaling gradients. One prediction of this model is that altering either gradient within the parasegment should alter cell fate choices in a consistent pattern within each segment of the AP axis. In fact, this has been shown to be true in the organization of the abdominal segments of the larval fly (reviewed in Hatini and DiNardo, 2001). At this stage of

development, the ventral epidermis of the abdomen clearly reveals a distinct pattern of alternating regions of smooth cuticle adjacent to areas containing outgrowths known as *denticles*. The binary decision regarding whether to make smooth cuticle or denticles depends on the activation of secondary signaling gradients initiated by the patterns of Wingless and Hedgehog within each parasegment. The genes required for denticle formation are activated in response to the Hedgehog gradient; smooth cuticle fates depend on the repression of those genes by actions of the Wingless pathway.

The segment polarity genes play a fundamental role in the compartmentalization of the fly embryo into developmental territories. After they have been formed, these units maintain the positional information provided by the parasegmental arrangements and the genetic programs activated by these interactions will govern cell fate choices within each segment.

D. Homeotic Selector Genes Establish Cell Fate Identity within Individual Segments

The final stage in the specification of the segmented body plan commences with the activation of the homeotic selector genes (Lewis, 1978). The members of this class control the developmental programs that determine the final cell fate decisions of each segment. Their importance for this final step of segmentation is clearly evident by the dramatic nature of their mutant phenotypes. The term *homeotic* refers to the transformation of one structure or segment into another. The fly mutant known as *Antennapedia*, in which the antennae in the fly head are transformed into a pair of legs, is a classic example of this phenotype (Lewis, 1978).

Drosophila was the first organism in which the genes responsible for homeotic transformations were identified. Currently, there are eight genes that have been characterized as homeotic selector genes based on these phenotypes. Each one codes for a transcription factor protein that contains a region known as the *homeodomain,* a DNA-binding motif of 60 amino acids that is evolutionarily conserved (reviewed in Lappin et al., 2006). The eight genes are collectively organized in a complex known as *Hom-C* (Lewis, 1978). Within this larger complex, the genes are divided into two regions referred to as the *Antennapedia* complex and the *bithorax* complex, respectively. The *Antennepedia* complex includes the genes *labial (lab), Antennapedia (Antp), sex combs reduced (scr), Deformed (dfd),* and *proboscipedia (pb)*; the genes of this complex are involved in the specification of head and thoracic segments (Kaufman et al., 1990). The *bithorax* complex consists of the three genes *Ultrabiothorax (Ubx), abdominal A (abdA),* and *Abdominal B (AbdB),* which are involved in the specification of abdominal segmental identities (Martin et al., 1995). There is a striking correlation between the organization of homeotic selectors genes within the fly genome and their transcriptional expression domains along the AP axis of the embryo and the adult fly (Lewis, 1978). The linear arrangement of the genes in the Hom-C complex correlates precisely with their expression domains along the AP axis. In fact, such spatial colinearity of Hox gene expression appears to be evolutionarily conserved among most species (Figure 9.6; reviewed in McGinnis and Krumlauf, 1992).

In the specification of *Drosophila* segment identity, the initiation of homeotic selector gene expression requires inputs from the gap gene proteins and the pair-rule proteins that control their expression within parasegmental domains. Gap and pair-rule proteins act through *cis*-acting regulatory regions,

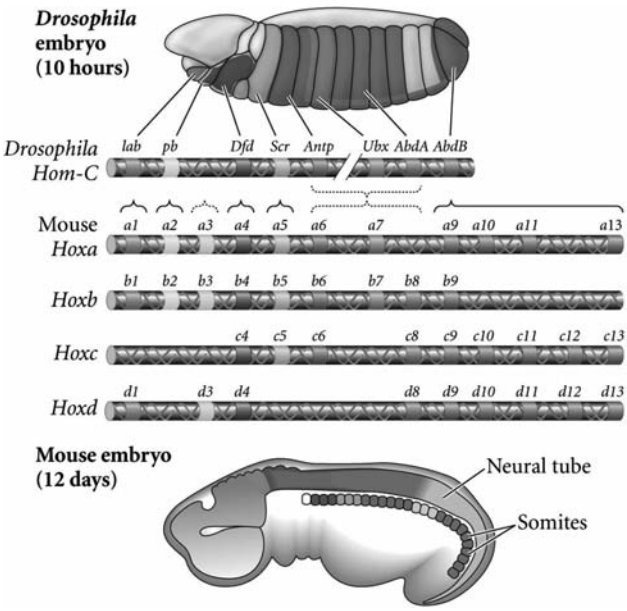

FIGURE 9.6 **The Hox gene family is evolutionarily conserved, as shown by the distinctive similarity between the Hom-C complex in *Drosophila* and the four *Hox* gene complexes in mouse.** In both species, the expression of these genes along the AP axis corresponds with their chromosomal orientation, a phenomenon known as *colinearity.* In the mouse, the four Hox clusters contain paralogous genes that are expressed in similar patterns. (Adapted with permission from Gilbert S: *Developmental Biology*, 8th ed. See color insert.)

referred to as *initiator enhancer elements,* to either activate or repress homeotic gene expression (reviewed in Maeda and Karch, 2006). For example, the gradients of Hunchback and Krüppel negatively regulate the expression of *abdA* and *AbdB* in parasegments that correspond with the head and thoracic regions (Casares and Sánchez-Herrero, 1995). The control of homeotic gene expression within each parasegmental population involves complex interactions that integrate the inputs (the specific constellations of gap and pair-rule proteins within those cells) at the level of these DNA regulatory regions (for an extensive review, see Maeda and Karch, 2006).

The dynamic expression patterns of the homeotic gene expression are further defined and stabilized along the AP axis by numerous mechanisms. As transcription factors, the proteins coded by the homeotic selector genes act to regulate the expression of each other. In general, posterior genes such as *Ubx, abdA,* and *AbdB* inhibit the expression of the more anterior homeotic selector genes in the posterior part of the embryo. For example, *Antp* expression in the abdominal segments is repressed by the combined activity of the Bithorax complex proteins (Harding et al., 1989; Gonzalez-Reyes and Morata, 1990). The absence of *Ubx* causes the posterior border of *Antp* to expand into the region normally expresses *Ubx*. This ectopic *Antp* expression contributes to the homeotic transformation of the third thoracic segment (no wings) into one with wings (resembling the second thoracic segment).

Homeotic selector genes are required for the specification of segmental identity during the larval and adult stages. Other mechanisms of gene regulation are required during these later stages, because those factors responsible for defining their early expression domains (the gap and pair-rule proteins)

are transient. How, then, do fly embryonic cells "fix" the state of homeotic gene expression to ensure continuity during later stages? The modification of chromatin structure appears to play a key role in the maintenance of homeotic selector gene expression. Two classes of genes—the Polycomb Group and the Trithorax Group—play opposing roles with regard to stabilizing homeotic gene expression. By remodeling chromatin structure into more compact states, the proteins of the Polycomb Group inactivate the *cis*-regulatory regions that control homeotic genes, thereby inhibiting their transcription during subsequent stages. By contrast, the Trithorax Group proteins appear to act collectively to keep chromatin in a state that favors transcriptional activation (reviewed in Simon and Tamkun, 2002).

Homeotic genes are responsible for regulating—either through transcriptional activation or repression—the specific genetic programs that direct cells within each segment toward their final cell fates. Nevertheless, two attributes of this class of genes have generated significant questions regarding how they can function in this capacity. The expression of homeotic selector genes is not confined to individual segments; most are expressed in a broader pattern along the AP axis. In addition, all homeotic selector genes share a highly conserved DNA-binding domain. This feature contributes to relatively weak binding specificity for an individual homeotic gene. In general, homeotic genes and their counterparts in other species require the activity of cofactor proteins to modulate their activity in a context-dependent manner (reviewed in Merabet et al., 2005; Moens et al., 2006). For example, one known target of homeotic selector proteins is the transcription factor Distal-less (Dll). Dll is required for the formation of legs in thoracic segments, and its expression is negatively regulated in abdominal segments by the actions of Ubx and AbdA. Cooperative DNA binding of the Dll repressor element by Ubx and AbdA involves two cofactors, Extradenticle and Homothorax, which act to improve the selectivity of DNA binding. In addition, recent work by Gebelein and colleagues (2004) suggests that segmentation genes Sloppy-paired and Engrailed also contribute to the contextual activity of homeotic selector proteins. These two cofactors do not influence DNA binding, but they appear to play a role in mediating the transcriptional repression by the homeotic selector proteins at the Dll repressor element (Gebelein et al., 2004, Merabet et al., 2005).

To date, relatively few targets of homeotic selector genes have been identified, although this will likely change with advances in genomics and proteomics. This area of research will continue to yield valuable insights into genetic and epigenetic mechanisms that control cell fate specification during fly embryogenesis.

III. CONCLUSION

By studying the development of *Drosophila melanogaster,* scientists have gained an unprecedented view into the cellular and molecular events that control the formation of the segmented body plan. Much can be learned about vertebrate development by examining the genes and gene products identified in this invertebrate model system. Many of the genes and gene pathways that function in the specification of the AP axis are highly conserved, which reflects the evolutionary connections among diverse organisms. The *Hox* gene family is a clear example of evolutionary conservation. As with flies, vertebrate Hox proteins play important roles in the patterning of the embryonic

body plan along the AP axis. In addition, vertebrate Hox genes are also essential to other processes, such as the formation of the limbs, hematopoiesis, and neurogenesis (Krumlauf, 1994). From a human perspective, a few congenital disorders have now been attributed to mutations of specific Hox genes. Synpolydactyly, which is a rare dominant disorder that affects digit formation, is caused by a mutation in *HOXD13* (reviewed in Goodman et al., 2001; Lappin et al., 2006). Another dominant disorder, hand–foot–genital syndrome, is caused by a mutation in *HOXA13*. Emerging research in the area indicates that the cofactors that mediate the context-dependent actions of fly homeotic genes (Exd, Hth) are also conserved in vertebrates. These include the PBC and the MEIS gene families; they likely have important developmental functions in addition to their roles in segmentation (reviewed in Moens et al., 2006).

Thus, the research into fly segmentation has clearly yielded significant insight into the genetic mechanisms involved in vertebrate development as well. With the advent of genomics and proteomics, scientists will likely uncover novel mechanisms of gene regulation in the specification of a segmented body plan.

SUMMARY

- The initiation of AP polarity begins during oogenesis and is based on the localization of maternal mRNAs such as *bicoid, oskar,* and *nanos*.
- The localization of maternal mRNAs involves complex molecular interactions with RNA-binding proteins, molecular motor proteins, and the cytoskeletal matrix. Translational repression plays a central role in regulating maternal gene expression.
- Gradients of Bicoid and Nanos pattern the anterior and posterior domains, respectively, within the early embryo. Bicoid has dual roles as both a translational inhibitor (of *caudal* mRNA) and as a transcriptional activator (of zygotic *hunchback* mRNA). The tyrosine kinase receptor Torso functions in the specification of the terminal regions of the embryo.
- Zygotic Hunchback becomes activated by the Bicoid gradient in a threshold-dependent manner. Together, Bicoid and Hunchback regulate the expression of the other gap genes necessary for head and thorax development. The specification of posterior cell fates requires the actions of Caudal.
- As transcription factors, gap proteins are involved in complex regulatory relationships that control the dynamic nature of their expression. Transcriptional repression by the gap proteins—either by mutual antagonistic interactions or asymmetrical inhibitions—is a central means of stabilizing gap expression patterns.
- Downstream targets of gap products include the pair-rule genes. Pair-rule genes are characterized by their striped expression patterns that reveal the parasegmental organization along AP axis. Enhancer elements within the promoters of pair-rule genes control the modular nature of their expression.
- Segment polarity genes become activated by the coordinated efforts of the pair-rule genes. The expression of Engrailed, Hedgehog, and Wingless define a signaling center that is created at compartmental boundaries. The signaling center at these boundaries provides cells with the positional

information and directs their development toward specific cell fates within each segment.

- Homeotic selector genes are responsible for establishing cell fate identity within individual segments. Mutations in these genes cause dramatic phenotypes in which one body part/segment (antennae) is converted into another body part/segment (leg). Gap gene proteins and pair-rule proteins initiate the expression patterns of the homeotic genes. As transcription factors, homeotic selector proteins in turn regulate the expression of other homeotic selector genes. In general, transcriptional repression is critical for these interactions whereby posterior homeotic selector proteins inhibit the expression of anterior homeotic selector genes.
- Homeotic selector genes are collectively organized as the Hom-C (the homeotic gene complex), which is located on the third chromosome. The Hom-C complex includes two regions: one is referred to as the *bithorax* complex, which includes three homeobox genes, and the other region is known as the *antennapedia* complex, which includes five homeobox genes. Their transcriptional expression patterns correspond with their organization within the Hom-C complex.
- The arrangement, expression, and activity of homeotic selector genes and their vertebrate counterparts (Hox) are highly conserved within the animal kingdom.

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GLOSSARY

5′, 3′ UTR (UnTranslated Region)
The specific regions of an mRNA transcript that are not translated and are often involved in the posttranscriptional control of gene expression.

Cytokinesis
The division of the cytoplasm after mitosis (nuclear division); the final act in the generation of two daughter cells from a single cell.

Enhancer
A sequence of DNA that interacts with specific regulatory proteins to control the rate and the efficiency of gene transcription. Enhancer elements influence the activation of promoter sequences located on the same chromosome. These regulatory regions can be located upstream (5′) or downstream (3′) of the gene, and some enhancers can be contained within intron sequences.

Epistasis
A genetic interaction involving the phenotypic suppression of a particular gene by another gene. In this way, a mutation in one gene can mask the mutant phenotype of another gene.

Morphogen
Any molecule (usually a transcription factor or a signaling molecule) that influences cellular behavior on the basis of concentration differences. Competent cells or tissues exhibit differential responses according to the concentration gradient of the morphogen.

Parasegments
Independent repeating units that exhibit gene-expression patterns that inform the formation of the segments.

Polyadenylation
The covalent addition of adenyl residues to the 3′ end of mRNA transcripts; a mechanism that controls the transport, stability, and translation of mRNA molecules.

Segmentation
The partitioning of the body plan into a series of morphologically similar units known as *segments*.

Syncytium
Any cell that contains many nuclei within a common cytoplasm. The fertilized fly egg develops in this manner, in which embryonic nuclei undergo a series of nuclear division in the absence of cytokinesis to generate a syncytial blastoderm.

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INTERNET RESOURCES ON *DROSOPHILA* DEVELOPMENT

Flybase
: http://www.flybase.org—A comprehensive database for genomic and molecular information about *Drosophila* (in conjunction with the Berkeley *Drosophila* Genome Project); the site also contains extensive links to other *Drosophila* Web sites, including *The Interactive Fly.*

Berkeley *Drosophila* Genome Project
: http://www.fruitfly.org—Up-to-date information about genomic information and other resources.

Bloomington *Drosophila* Stock Center
: http://flystocks.bio.indiana.edu/bloomhome.htm—Fly strains that are commercially available to the scientific community.

Flymove
: http://flymove.uni-muenster.de/—Site featuring information about *Drosophila* development, including tutorials, media, and other material.

Yale Developmental Gene Expression
: http://genome.med.yale.edu/Lifecycle/

Flymine
: http://www.flymine.org—Site containing extensive genomic information about *Drosophila* and other organisms and that provides opportunities for comparative analyses across different genomes.

10 ANTERIOR–POSTERIOR PATTERNING IN MAMMALS

SIGOLÈNE M. MEILHAC

Department of Developmental Biology, Pasteur Institute, Paris, France

INTRODUCTION

The anterior–posterior (AP) axis is the main body axis of mammals. It is defined along the head, trunk, and tail of the adult, and it is set up in several steps (Idkowiak et al., 2004; Stern et al., 2006). Polarities in the early conceptus may be observed that anticipate the future poles and the direction of the axis, but they are not strictly correlated. A symmetry-breaking event establishes the orientation of the axis and fixes its direction. The axis is formed when irreversible morphologic features are detected in the conceptus, and it is only later that the axis is established, when embryonic cells are being assigned a particular fate for a particular position on the axis, under the control of an organizer. This latter process is initiated at the time of gastrulation (see Chapters 9 and 40).

AP patterning has been best characterized in amphibian embryos (see Chapters 9 and 12), in which maternal factors, already present in the unfertilized egg, play an essential role. By contrast, the role of maternal factors in the mouse is less clear. The early mouse conceptus has more plasticity, and it can recover from many kinds of experimental manipulations, such as ablation or aggregation. This indicates that polarity can only be a bias during early stages and that it is not fixed until later stages. Therefore, it is controversial whether polarity in the early mouse conceptus is important for AP patterning (Rossant and Tam, 2004; Zernicka-Goetz, 2005; Gardner, 2005). In addition, the mammalian egg gives rise to extra-embryonic structures such as the placenta, which are necessary for the nutrition and protection of the embryo, but which also interact with embryonic tissues. This therefore affects the context of AP patterning in mammals.

From an historic point of view, we owe the concept of embryonic axes to von Baer, which was based on his observations of the frog embryo in 1834 (Brauckmann and Gilbert, 2004). Almost a century later, Spemann and

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Mangold (1924) demonstrated the existence of an organizer that induces a secondary axis when grafted ectopically. In 1875, Rauber was the first to identify the primitive streak in mammals, which corresponds with the first morphologic sign of a posterior pole. For a long time, it has been thought that AP patterning was a consequence of gastrulation and that only products of the primitive streak bestow a pattern on the embryo. A major discovery was made by Beddington (1994), who showed that an organizer also exists in mammals. The node was originally described by Hensen (1876) as a morphologic entity in the rabbit embryo. Beddington showed that, in the mouse, the node functions as an organizer equivalent to that described by Spemann in amphibians, because it induces a secondary axis when grafted ectopically. However, the induced axis is incomplete and lacks the head. For the induction of the head, another structure is required, and this is referred to in the mouse as the *anterior visceral endoderm* (AVE; Thomas and Beddington, 1996). The AVE lies within an extra-embryonic tissue, which indicates that reciprocal interactions between embryonic and extra-embryonic tissues are required for AP patterning of the mammalian embryo. The AVE is not only required for the establishment of the anterior of the body axis during gastrulation but also, before this, to position the primitive streak at the posterior pole and to break the symmetry of the implanting conceptus.

In this chapter, I shall deal mainly with the mouse, and divergences among mammals will be discussed at the end. AP patterning becomes visible in the mouse at the time of implantation into the uterus (Figure 10.1). The development of the mouse conceptus at this stage is complex. Before implantation, 3.5 days after fertilization, the blastocyst is initially composed of two lineages: the trophectoderm, which will contribute to the placenta, and the inner cell mass, from which the totipotent embryonic stem cells derive. After implantation, the inner cell mass segregates into the epiblast lineage, which will form all adult tissues, and the primitive endoderm, which will contribute to the visceral and parietal yolk sacs. Major morphologic changes occur on embryonic day 4.5, leading to the transformation of the blastocyst into the egg cylinder. In addition to an embryonic region, where the epiblast lies, the egg cylinder contains an extra-embryonic region that includes a derivative of the trophectoderm called the extra-embryonic ectoderm (ExE). Visceral endoderm covers the whole egg cylinder, but it may have different characteristics in either region. It is at the stage of the egg cylinder that AP polarity becomes visible. The ExE and the visceral endoderm are the two major extra-embryonic tissues that play essential roles in specifying the polarity of the underlying epiblast, which constitutes the embryo.

How is the symmetry of the egg cylinder broken? On embryonic day 5.5, no morphologic sign of AP polarity is visible (see Figure 10.1). The embryonic day 5.5 egg cylinder is polarized along the proximal–distal axis, but, from a molecular point of view, it is symmetrical around its circumference. However, a group of visceral endoderm cells at the distal tip have been specified and are referred to as the distal visceral endoderm (DVE). They express a number of genes, such as the homeobox gene *Hhex* (Thomas et al., 1998), as well as *Lefty1* (Meno et al., 1996; Yamamoto et al., 2004) and *Cer1* (Belo et al., 1997), which encode antagonists of Nodal and bone morphogenetic protein (BMP) signaling that play key roles in restricting posterior identity and the formation of the primitive streak (Perea-Gomez et al., 2002). DiI labeling has demonstrated that DVE cells move asymmetrically toward one side of

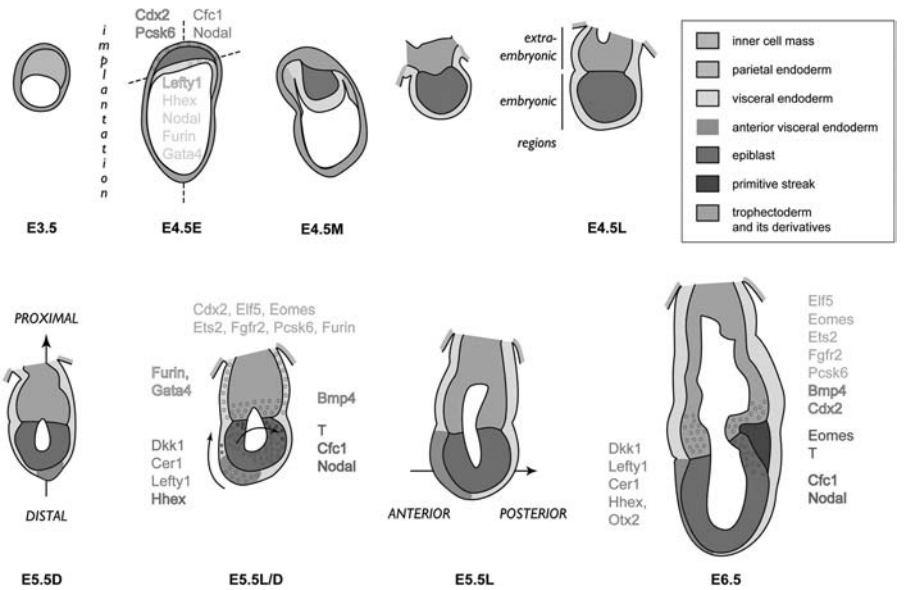

FIGURE 10.1 **Peri-implantation development of the mouse embryo.** Stages of development of the mouse embryo expressed as number of days after fertilization from the period of implantation into the uterus until gastrulation. The tissues are drawn as distinct colors. From E4.5M, the egg cylinder is shown without the parietal yolk sac (mural trophectoderm and parietal endoderm). The asymmetry of the implanting blastocyst is shown at E4.5E as a "tilt" of the embryonic (top)/abembryonic (bottom) axis (vertical dashed line) in relation to the plane of the primitive endoderm (oblique dashed line). The embryonic day 3.5 blastocyst is transformed into an egg cylinder around embryonic day 4.5 (substages E, M, or L as early, mid or late). It includes an embryonic region composed of epiblast and visceral endoderm and an extra-embryonic region composed of extra-embryonic ectoderm (a trophectoderm derivative) and visceral endoderm. The expression of some genes important for anterior–posterior patterning is schematized with dots (unless ubiquitous in a given tissue) and indicated on the sides. *Otx2,* which encodes an homeodomain transcription factor, is also expressed on embryonic day 5.5, including in the epiblast, and it has not been represented for clarity. Upon migration of the anterior visceral endoderm on embryonic day 5.5, the transformation of the proximal–distal axis into an anterior–posterior axis is shown by arrows (substages *D, L/D,* and *L* as distal, laterodistal, and lateral visceral endoderm thickening, respectively).

the embryo (Thomas et al., 1998). Mutant phenotypes, such as $Otx2^{-/-}$ (Kimura et al., 2000) and $Smad2^{-/-}$ (Waldrip et al., 1998), have shown that this movement is essential for AP patterning. This rotation of an initial proximal–distal polarity is a major event that breaks the symmetry of the implanting conceptus and leads to the formation of the AP axis. At this time, when the anterior pole is defined, cells derived from the DVE have moved and now become the AVE. Conceptuses on embryonic day 5.5 can be staged according to the position of DVE or AVE cells (E5.5D, E5.5L/D, and E5.5L, according to Rivera-Pérez et al., 2003; see Figure 10.1). The molecular network underlying the formation of the AP axis in the mouse is centered on the transforming growth factor (TGF)-β–related signal Nodal (reviewed in Ang and Constam, 2004). Nodal is required in the epiblast to specify the AVE (Brennan et al., 2001). Nodal is ubiquitously expressed, and its gradient of activity is determined by other factors, such as proteins secreted from the AVE or proteases (Pcsk6 [also known as Spc4] and Furin), which are required to produce the mature and active form of Nodal. The secretion of proteases

by the ExE results in a gradient of Nodal, with higher activity proximal to the ExE than at the distal tip (Beck et al., 2002). The patterns of expression of some genes involved in AP patterning are summarized in Figure 10.1. These genes are not very different from those involved in AP patterning in other vertebrate species, but the interplay between their products is different, and so are the embryonic structures that generate them.

In this chapter, I concentrate on recent findings that have refined our understanding of the formation of the AP axis within the mouse embryo at the time of implantation. First, I consider how the AVE is defined by markers and how it functions as a symmetry-breaking event. Second, I outline how AVE cells are specified by the inhibitory role of the ExE. Finally, I discuss the lineage of AVE cells and the signs of polarity that precede their appearance within the visceral endoderm. I begin by describing later events, because these are better characterized than the events of the earlier stages.

I. A HETEROGENEOUS POPULATION OF CELLS DEFINES THE ANTERIOR POLE

A. Markers of the Anterior Visceral Endoderm

Numerous markers are now used to define the AVE. Originally the AVE was characterized by the expression of the homeobox gene *Hesx1* on embryonic day 6.5 as well as by its ability to induce anterior ectoderm and neurectoderm during later stages (Thomas and Beddington, 1996). Markers of the AVE have a dynamic expression pattern that follows the migration of these cells. Some markers are detected early (in the DVE on embryonic day 5.5), including the homeodomain transcription factor Hhex (Thomas et al., 1998), the TGF-β antagonists Cerberus-related 1 (Cer1; Belo et al., 1997) and Lefty1 (Yamamoto et al., 2004), and the Wnt antagonist Dkk1 (Kimura-Yoshida et al., 2005). The genes encoding these factors are expressed in overlapping domains, but a detailed analysis of coexpression by double *in situ* hybridization highlighted that some cells express *Lefty1* but not *Hhex* (see Figure 10.1; Yamamoto et al., 2004). Similarly, *Dkk1* is expressed in more proximal cells at E5.5D and in more anterior cells later (Kimura-Yoshida et al., 2005). In addition, transgenic green fluorescent protein (GFP) alleles, which offer a cellular resolution of the expression patterns, have shown that markers such as *Hhex* may not be restricted to neighboring cells but that rather they may be expressed in a salt-and-pepper pattern (Figure 10.2, A; Srinivas et al., 2004). Therefore, the repertoire of AVE-specific genes may be variably expressed among cells located in an AVE position.

Quantitative variability in gene expression may supplement this qualitative variability. On the basis of transgenic marker expression, it would appear that the DVE is composed initially of about 10 to 15 cells (*Hhex-GFP;* Srinivas et al., 2004), whereas, after migration, about 30 cells can be counted in the AVE (*Cer1-GFP;* Richardson et al., 2006). Considering that AVE cells proliferate at a low rate (described later), these observations suggest that *de novo* or increased expression of AVE markers occurs in the AVE.

In addition to their molecular signature, AVE cells display a characteristic morphology. Unlike other visceral endoderm cells in the embryonic region, which are squamous, AVE cells have a columnar shape. Confocal microscopy indicates that columnar cells are polarized and that they contain many

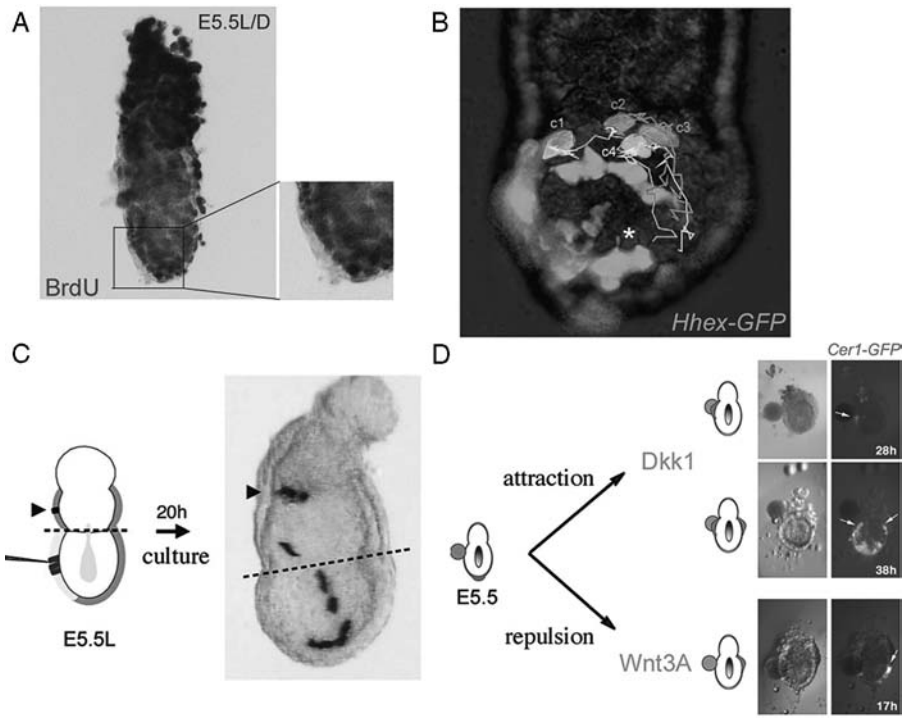

FIGURE 10.2 **Polarized movement of the anterior visceral endoderm (AVE). A,** The lower rate of proliferation of AVE cells detected by BrdU labeling suggests that these cells may be displaced forward by the posterior dividing cells. (Reprinted by permission from Macmillan Publishers Ltd. *Nature,* Yamamoto et al., copyright 2004, http://www.nature.com.) **B,** Tracking of AVE cells by time-lapse imaging of *Hhex-GFP* transgenic embryos indicates active migration. The asterisk shows a cell protrusion in the direction of migration. Trajectories of individual cells from embryonic day 5.5 conceptuses cultured for 10 hours are represented in colors. (Reproduced by permission of Srinivas et al., 2004.) **C,** Growth of clones of AVE cells is oriented as a crescent. Cells are labeled with horseradish peroxidase. (Adapted from figure 6, A and C-E from *Dev Biol*, vol 261, Rivera-Pérez JA et al., Dynamic morphogenetic events charaterize the mouse visceral endoderm, 470–487, copyright 2003, with permission from Elsevier.) **D,** Wnt signaling appears to affect the direction of migration of distal visceral endoderm cells as assessed by culture of *Cer1-GFP* transgenic embryos in the presence of soaked beads. Dkk1 at E5.5D may determine where the anterior pole will form (upper lane) or attract a few fluorescent cells when implanted on the posterior pole slightly later at E5.5L/D (middle lane). Wnt3A repels fluorescent cells and thus prevents the formation of the anterior pole. (Adapted from figure 8, B from *Dev Cell*, vol 9, Kimura-Yoshida et al., Canonical Wnt signaling and its antagonist regulate anterior-posterior axis polarization by guiding cell migration in mouse visceral endoderm, 639–650, copyright 2005, with permission from Elsevier. See color insert.)

vacuoles at the apical pole, whereas the large nucleus is localized at the basal pole facing the epiblast (Rivera-Pérez et al., 2003; Srinivas et al., 2004). This morphologic feature, which is referred to as *visceral endoderm thickening,* correlates with the distribution of AVE markers such as *Hhex,* although rare cells may be columnar and *Hhex* negative (and vice versa). This illustrates again that markers of the AVE incompletely overlap.

B. Movement of Anterior Visceral Endoderm Cells

Two possible mechanisms of AVE movement have been proposed. The differential growth of the visceral endoderm cells may displace AVE cells. Alternatively, the active migration of AVE cells may be the driving force. These

hypotheses, which may be compatible, have been tested, together with the investigation of signals that may direct the polarized movement of DVE cells.

In accordance with the first mechanism, AVE cells have been shown to proliferate at a low rate during their migration based on BrdU labeling (see Figure 10.2, A). An elegant approach using Lipofectamine™ to transfect cells *in vivo* showed that the ectopic expression of *Lefty1* and/or *Cer1* in nonanterior visceral endoderm cells drives the movement of DVE cells toward the transfected cells, whereas ectopic *Nodal* expression has the opposite effect. This is coupled with an effect on cell proliferation, which is promoted by Nodal and inhibited by its antagonists. The ectopic expression of the gene that encodes the cyclin-dependent kinase Cdk2, which is essential for cell cycle progression, also repels DVE movement when Nodal is present (wild-type background; Yamamoto et al., 2004). Taken together, these results suggest that differential cell proliferation is important for the anterior movement of cells. Nodal signaling directs movement such that cell proliferation is inhibited by Nodal antagonists in the AVE.

In the context of the second mechanism, time-lapse imaging of *Hhex-GFP*–expressing cells in cultured embryos showed that AVE cells have morphologic properties that are characteristic of migratory cells, including protrusions (Figure 10.2, B). Migration occurs in two steps. First, AVE cells migrate away from the distal tip toward the proximal pole of the conceptus. However, when they reach the embryonic/extra-embryonic junction, their migration is reoriented laterally, as if it was transitorily blocked by the junction (Thomas and Beddington, 1996; Srinivas et al., 2004). In agreement with this, AVE clones of cells labeled at E5.5L are oriented as a crescent (Figure 10.2, C; Rivera-Pérez et al., 2003).

Movement of the DVE not only depends on Nodal signaling but also on Wnt signaling. This is asymmetric in the pregastrulating visceral endoderm. In addition to the expression of the gene that encodes the Wnt-antagonist Dkk1 at the leading edge of the AVE, the Wnt effector β-catenin is enriched in the posterior visceral endoderm. Interestingly, $Otx2^{-/-}$ mice, in which DVE migration does not occur, may be rescued by the inhibition of Wnt signaling, either with increased *Dkk1* or reduced *β-catenin*. In embryo culture, beads soaked with Dkk1 attract AVE cells, whereas they repel cells when they are soaked with a low dose of Wnt3A (Figure 10.2, D; Kimura-Yoshida et al., 2005). These experiments indicate that Wnt signaling is also involved in directing DVE migration.

In conclusion, the movement of the AVE appears to involve active cell migration as well as differential rates of cell proliferation. It is regulated by Nodal and Wnt signaling. Various criteria are used to monitor the AVE, and it is notable that they do not overlap completely. This highlights the complexity of this signaling center, and it raises the question about how it is specified.

II. THE ANTERIOR VISCERAL ENDODERM DOMAIN IS RESTRICTED BY THE EXTRA-EMBRYONIC ECTODERM

The trophectoderm is required for implantation of the embryo into the uterus. Factors such as the homeodomain transcription factor Cdx2, the T-box transcription factor Eomes, and Fgf signaling via the Fgfr2 receptor regulate the correct differentiation of trophectodermal cells. After implantation,

trophectoderm derivatives such as the ExE are also required for correct AP patterning of the mouse embryo. This has been demonstrated by genetic approaches. *Ets2* (Georgiades and Rossant, 2006) and *Elf5* (Donnison et al., 2005), which encode transcription factors of the erythroblast transformation specific (ETS) family, are expressed in the ExE. The inactivation of either of these genes abolishes the formation of the ExE, and markers of this tissue, such as *Cdx2* and *Bmp4,* are absent. Although *Ets2* is also expressed in the ectoplacental cone, its inactivation does not completely inhibit the formation of this structure, which can still signal to the visceral endoderm and the epiblast and compensate for the absence of the ExE. In both mutants, induction of the DVE takes place. However, in the most strongly affected $Ets2^{-/-}$ conceptuses (and possibly also in $Elf5^{-/-}$), the anterior movement of the DVE is impaired such that markers of the AVE are expanded and incompletely

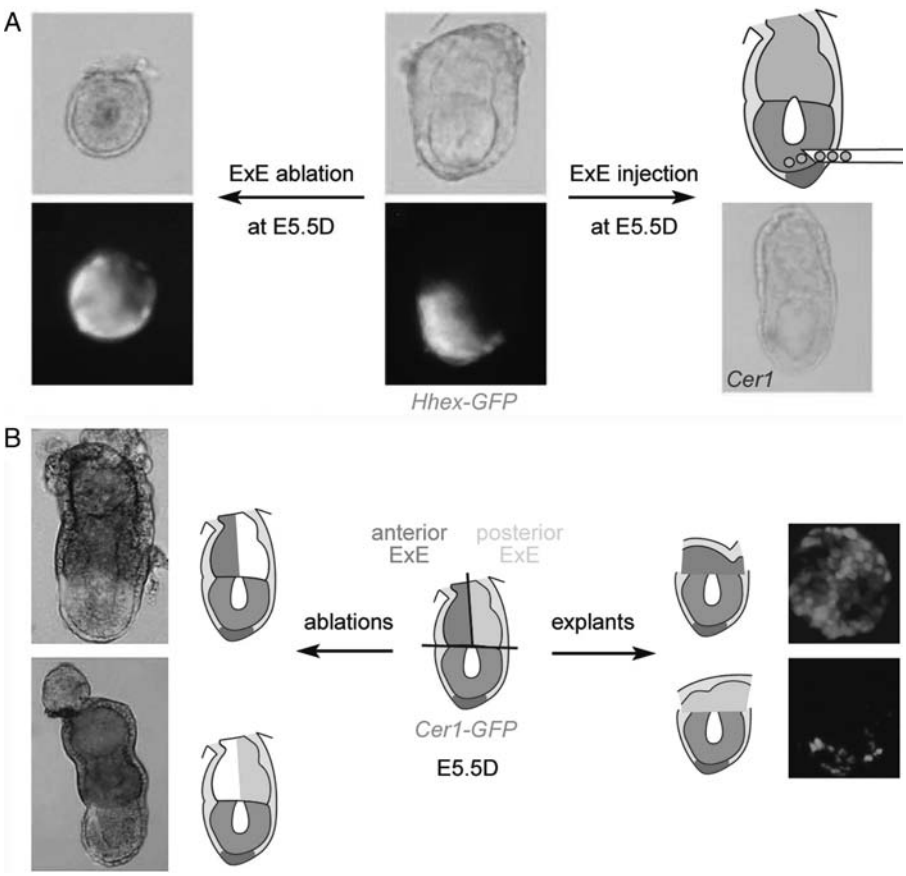

FIGURE 10.3 **Role of the extra-embryonic ectoderm (ExE) in repressing anterior visceral endoderm markers. A,** Ablation and injection of cells of the ExE show the inhibitory role of the tissue on anterior visceral endoderm markers such as *Hhex* (transgenic line) and *Cer1* (*in situ* hybridization). Conceptuses are shown after overnight culture. (Adapted by permission of Rodriguez et al., 2005.) **B,** Selective ablation or transplantation of regions of the ExE shows that the inhibitory activity is contained within the posterior ExE. Conceptuses are shown after 18 hours in culture. (Adapted from figures 1, C and D, and figure 4, F from Mech Dev, Vol 123, Richardson L et al., Regionalised signaling within the extraembryonic ectoderm regulates anterior visceral endoderm positioning in the mouse embryo, 288–296, copyright 2006, with permission from Elsevier. See color insert.)

displaced anteriorly. *Ets2* and *Elf5* are also required later, as is *Bmp4* (Winnier et al., 1995), for gastrulation and mesoderm formation.

Recent embryologic approaches have provided additional insights into the role of the ExE in AP patterning. The ablation of the ExE at E5.5D leads to the expansion of AVE markers (Figure 10.3, A; Rodriguez et al., 2005; Richardson et al., 2006). Reciprocally, the ectopic injection of ExE cells represses AVE markers. This shows that interaction with the ExE is essential to prevent proximal visceral endoderm cells from adopting an AVE identity. This is a transitory interaction, because later ablation has no such effect. In addition, ExE promotes posterior identity as indicated by the loss of markers such as the T-box transcription factor T (also known as brachyury) in ablated conceptuses. The selective ablation or transplantation of regions of the ExE has demonstrated that the repressing signal for AVE formation is restricted to the posterior half (Figure 10.3, B; Richardson et al., 2006). This indicates that the ExE is functionally regionalized, although the molecular basis of the difference between anterior and posterior regions remains to be determined.

The nature of the ExE signal remains to be understood. A good candidate is Bmp4, which is a secreted protein of the TGF-β family that is expressed in the ExE at the time of AVE formation (see Figure 10.1). In addition, knockdown of Bmp4 by RNA interference at peri-implantation stages impairs the expression of the AVE marker *Cer1,* which suggests the disruption of AP patterning (Soares et al., 2005). Another candidate for mediating the effect of the ExE is Nodal signaling, which is required for AVE induction. Although Nodal is expressed throughout the epiblast, it is processed into an active form by proteases such as Pcsk6, which are secreted by the ExE (Beck et al., 2002). This may provide a gradient of Nodal activity in the proximal–distal axis of the epiblast. The ectoplacental cone also secretes Nodal proteases and may compensate for the absence of ExE. This may explain why the phenotype of *Ets2* and *Elf5* inactivation is less severe than that of ablation experiments. Bmp4 and Nodal signaling are not independent, because *Bmp4* is induced by Nodal and, in turn, Bmp4 induces the gene encoding Nodal coreceptor Cfc1, which is also known as cripto (Ang and Constam, 2004).

In conclusion, the ExE (and, principally, its posterior half) plays a crucial role not only in restricting the induction of anterior markers but also in inducing posterior markers, thus leading to mesoderm formation. However, the nature of the signal is unclear.

III. ORIGIN OF THE ANTERIOR VISCERAL ENDODERM: WHERE DO THESE CELLS COME FROM?

A. Tracing Anterior Visceral Endoderm Precursors

Do AVE cells represent a distinct lineage? Markers of the AVE have been used to trace its origin. *Lefty1,* for example, is already expressed in the inner cell mass of the blastocyst on embryonic day 3.5 and, after implantation, it continues to be expressed in a few cells of the primitive endoderm as it begins to form (see Figure 10.1; Takaoka et al., 2006). However, there may be a re-expression of molecular markers, so it cannot be assumed that the progeny of early *Lefty1*-expressing cells contribute to the AVE later. In addition, AVE markers also show heterogeneity in their expression at this early stage. On embryonic day 4.5, *Hhex* appears to be more broadly expressed than *Lefty1*

throughout the primitive endoderm (Thomas et al., 1998). These conditions indicate that it may be misleading to conclude that the early expression of AVE markers defines AVE precursors.

Tracing the clonal origin of AVE cells is a delicate matter, because implantation cannot be fully reproduced *in vitro*, and conceptuses can only be cultured over a short period. This has been analyzed by a cell-labeling approach. Single precursors of the visceral endoderm were targeted before implantation, at the blastocyst stage, by the microinjection of mRNA encoding a fluorescent protein (Figure 10.4, A). Positive blastocysts were transferred into the uterus of a foster mother to permit the correct development of the conceptuses over a long period of time. The progeny of an injected cell, which forms a clone, was analyzed after implantation on embryonic day 5.5. This experiment has shown that the domain expressing the *Cer1-GFP* transgene is composed of a few clonally related cells (Figure 10.4, B and C; Perea-Gomez and S.M.M., Piotrowska-Nitsche, Gray, Collignon and Zernicka-Goetz, unpublished observations). This indicates that the AVE has a polyclonal origin, that it is derived from more than one precursor between embryonic days 3.5 and 5.5. However, this does not address the question of whether and when some precursors become restricted to the AVE and thus segregate from the rest of the visceral endoderm cells. Using time-lapse microscopy, *Cer1*-expressing cells have been monitored in cultured transgenic conceptuses

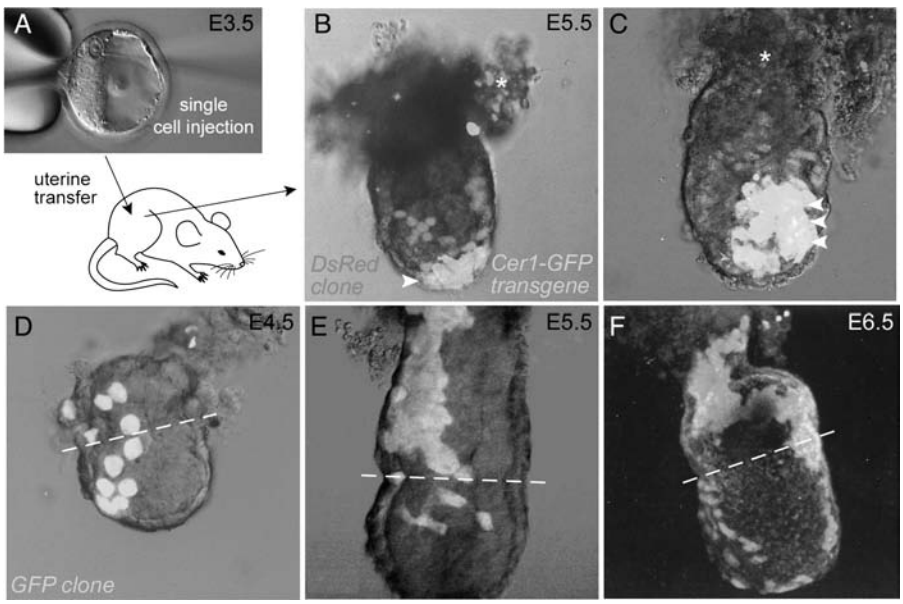

FIGURE 10.4 **Origin and regionalization of the visceral endoderm. A,** Approach for labeling visceral endoderm precursors in the blastocyst by the microinjection of mRNA encoding a fluorescent protein. The injected cell is highlighted in red. Positive embryos are transferred into a recipient uterus for further development. **B** and **C,** Examples of clones of red-positive cells in the *Cer1-GFP* transgenic line showing the polyclonal origin of anterior visceral endoderm cells. Arrowheads point to the double-positive cells; asterisks indicate background fluorescence in the parietal endoderm. **D, E,** and **F,** Examples of clones of green-positive cells on embryonic days 4.5, 5.5, and 6.5 showing that cell dispersion becomes specific to the embryonic region around embryonic day 5.5. The junction between the extra-embryonic (top) and embryonic (bottom) regions is represented by a dashed line. (Perea-Gomez and S.M.M., Piotrowska-Nitsche, Gray, Collignon and Zernicka-Goetz, unpublished data. See color insert.)

sequentially from E4.5M for 12 hours and from embryonic day 5.25 for 9 hours. These experiments indicate that the AVE has a double origin. It is formed by cells that have initiated *de novo* expression of *Cer1* at the distal tip of the egg cylinder as well as by the progeny of cells that expressed *Cer1* already in the E4.5M conceptus (Torres-Padilla and Richardson, Kolasinska, S.M.M., Luthe-Eversloh, and Zernicka-Goetz, unpublished observations). These results suggest that the AVE derives from several precursors that start expressing AVE markers at more than one stage. Thus, it may be possible that AVE cells are induced continuously over a period of time. How this takes place remains to be investigated. How are AVE genes regulated? Do they control each other's expression?

B. Polarity and Regionalization of the Visceral Endoderm

Relevant to the mechanism of induction of AVE cells is the investigation of earlier signs of polarity in the visceral endoderm, which may influence the formation of the AVE. Early expression of *Lefty1* (Takaoka et al., 2006) and *Cer1* (Torres-Padilla and Richardson, Kolasinska, S.M.M., Luthe-Eversloh, and Zernicka-Goetz, unpublished observations) indicates asymmetry in the primitive endoderm as it begins to form. This is reminiscent of a morphologic feature of the blastocyst at implantation, known as "tilt." The axis of the blastocyst, from the inner cell mass to the blastocele (the embryonic–abembryonic axis), does not lie perpendicular to the surface of the inner cell mass, where the primitive endoderm forms (Smith, 1985). It has been proposed that this morphologic asymmetry correlates with the later AP axis (Smith, 1985), thereby defining its orientation but not its polarity (Gardner et al., 1992). *Lefty1* expression is localized on the "upper" side of the inner cell mass, closer to the embryonic pole (represented in Figure 10.1 at E4.5E). It remains to be seen whether this early polarity plays a causative role in driving later axial patterning.

In the egg cylinder, regionalization of the whole visceral endoderm has been analyzed using the same microinjection approach as described previously. Precursors of the visceral endoderm were labeled in the blastocyst, and the distribution of labeled cells was analyzed at postimplantation stages, including on embryonic days 4.5 (Figure 10.4, D), 5.5 (Figure 10.4, E), and 6.5 (Figure 10.4, F). This study has revealed that labeled visceral endoderm cells have a coherent behavior until embryonic day 4.5 and that they usually do not spread throughout the whole tissue (Perea-Gomez and S.M.M., Piotrowska-Nitsche, Gray, Collignon and Zernicka-Goetz, unpublished observations). From the time of the migration of the AVE at E5.5L, distinct patterns of labeled cells are observed in the embryonic and extra-embryonic regions. Labeled cells are dispersed within the embryonic region, whereas they remain close neighbors in the extra-embryonic region. This regionalization of the mode of growth of visceral endoderm cells is thus concomitant with or a consequence of the emergence of the first morphologic signs of AP polarity. However, such regionalization may relate to earlier asymmetries of the embryo, because, in the blastocyst, the precursor cell closer to the polar body tends to contribute more cells to the embryonic region of the visceral endoderm (Weber et al., 1999). This regionalization of the visceral endoderm depends on Nodal signaling. On embryonic day 4.5, genes encoding Nodal and its protease Furin are expressed in the primitive endoderm (see Figure 10.1). The analysis of mutant embryos indicates that early Nodal signaling is required

to specify the embryonic region of the visceral endoderm. Furthermore, explant cultures show that early Nodal signaling in the embryonic region of the visceral endoderm provides an instructive signal for DVE induction. This becomes efficient when the growth of the egg cylinder places potential DVE cells beyond the range of inhibitory signals from the ExE (Mesnard et al., 2006). The regionalization of the visceral endoderm into embryonic and extra-embryonic domains therefore appears to be a prerequisite for AVE induction and further AP patterning.

IV. EVOLUTIONARY PERSPECTIVE

Variations of the mouse model are observed in other mammals (Eakin and Behringer, 2004). Blastocysts have a similar structure except in cases such as marsupials, when the blastocyst is unilaminar without an inner cell mass. It is not always at this stage that implantation in the uterus occurs, because, in marsupials and artiodactyls, gastrulation is complete before implantation. An extreme case is the horse conceptus, which does not implant until after the limb buds are visible. This shows that, in many cases, AP patterning is intrinsic to the conceptus and independent of interactions with maternal tissues. Even in the mouse, in which AP patterning occurs at the time of implantation, it has been shown that the emergence of the AP axis does not correlate with the axes of the uterus (Mesnard et al., 2004). The development of the blastocyst into an egg cylinder, including a cup-shaped epiblast, is peculiar to rodents. This permits a dramatic reduction in the volume of the conceptus (up to 50-fold) for embryos of similar size and thus an increase in the size of the litter. By contrast, the human gastrula (like that of most other mammals) remains unfolded, and the epiblast displays a planar morphology as a disc. This variation implies geometric changes such that the proximal–distal axis of the mouse egg cylinder becomes translated into a peripheral–central radius of the disc. Extra-embryonic tissues are present in all mammals, but their structures may differ, raising the question of how they interact with the epiblast. The equivalent of the ExE in nonrodent mammals is unclear. In species such as the guinea pig, the epiblast dissociates from trophectoderm derivatives, thereby preventing interactions between the tissues. In other cases, such as marsupials and ungulates, the trophectoderm covering the inner cell mass (or Rauber's layer), from which the ExE derives in mouse, disappears; this leads to the exposure of the inner cell mass to the exterior. In this case, we can only speculate about the tissue equivalent to the ExE. It could be the remaining trophectoderm, which surrounds the epiblast. The other extra-embryonic structure essential for AP patterning in the mouse, the AVE, may be conserved in mammals, although further characterization is required. An equivalent has been described in the rabbit, and it is known as the *anterior marginal crescent*. Similar to the mouse AVE, it is composed of columnar cells, which move. It is functional for the induction of mesoderm and of neural tissues later, and gradients of gene expression, including that of *Cer1* and *Dkk1,* are compatible with the mouse model (Idkowiak et al., 2004). In humans, a thickening of the prospective anterior endoderm has been observed, which is morphologically similar to the mouse AVE; however, its function is unknown.

Similarities to the mouse model can be identified not just in mammals but also in other vertebrates. In the chick, the posterior marginal zone is necessary

to induce the primitive streak (Stern, 2004). This is mediated by the TGF-β signal Vg1 and by cWnt8c, which in combination activate Nodal in the epiblast. Cells of the posterior marginal zone do not contribute to adult tissues, and they are localized at the periphery of the area pellucida epiblast, which later forms adult tissues. This position of an extra-embryonic tissue, in addition to its early role as a Nodal inducer, suggests that the posterior marginal zone may be equivalent to the mouse ExE. An equivalent to the mouse AVE in the chick is the hypoblast, an extra-embryonic tissue that covers the epiblast and expresses similar markers, including *Hesx1, Hhex, Otx2,* and *Cerberus*. The hypoblast is also displaced, as is the mouse AVE, leading to the removal of Nodal inhibition and the induction of the primitive streak. However, the movement of the hypoblast is not intrinsic; rather, it results from the growth of another tissue, the endoblast. In other vertebrates, AVE-like cells are also observed, such as the yolk cells of the vegetal region in the frog and the dorsal yolk syncytial cells in the zebrafish. These cells are mobile, express *Cerberus* or *Hhex,* and are important for AP patterning.

V. CONCLUSIONS

A combination of genetic, cellular, and embryologic experiments has been necessary to unravel the formation of the AP axis in mammals. The mouse remains the model of choice, because it is more amenable to all three kinds of manipulation and easily bred under laboratory conditions. AP patterning depends on an interplay between embryonic and extra-embryonic tissues, and it is centered on the specification and directed movement of the AVE. Key molecules involved in this process include Nodal and Wnt signaling pathways as well as specific transcription factors. A number of important questions now need to be answered: How is the heterogeneous gene repertoire of AVE cells specified? What is the nature of the inhibitory signal from the extra-embryonic ectoderm? How do early asymmetries in the preimplantation embryo relate to later AP patterning? Such basic research provides a framework in which clinical abnormalities can be understood, because it is probable that human AP patterning follows similar principles to that of the mouse.

SUMMARY

- Polarized movement of the distal visceral endoderm is a central event for the AP patterning of the mouse embryo, because it breaks irreversibly the symmetry of the egg cylinder.
- Movement of the AVE appears to be driven by both differential cell proliferation and active cell migration under the control of Nodal and Wnt signaling.
- Several markers of the AVE have now been identified, but their incomplete overlap raises the questions of how AVE cells are specified and how the expression of their heterogeneous gene repertoire is regulated.
- The extra-embryonic ectoderm emerges as an important regulator of AVE specification, particularly its posterior half. It will be interesting to determine the factors responsible for its regionalized activity.

- Other asymmetries have been characterized before DVE migration, including morphologic features and regionalized gene expression, but their involvement in AP patterning remains unknown.
- The AVE has a polyclonal origin. The specification of AVE cells does not appear to occur at a single stage.
- The mouse model may not fully apply to other mammalian species. The AVE has an equivalent in the rabbit and also in other vertebrate species. However, the equivalent of the extra-embryonic ectoderm is unclear.

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GLOSSARY

Anterior visceral endoderm (AVE)
This group of cells, which arises from extra-embryonic tissue, is the first morphologic sign of the anterior pole after embryonic day 5.5, and it functions to restrict posterior markers and later to induce neural fate. AVE cells, like other visceral endoderm cells, later contribute to the yolk sac.

Artiodactyls
A group of herbivorous mammals with even-toed hoofs. This includes pigs, hippopotamuses, and ruminants.

Conceptus
Embryonic (epiblast) and extra-embryonic tissues, all derived from the zygote.

Distal visceral endoderm (DVE)
This group of cells is specified at the distal tip of the egg cylinder on embryonic day 5.5. As it migrates towards one side, the anterior pole is defined, and the group of cells is referred to as the *anterior visceral endoderm*.

Egg cylinder
The mouse conceptus after implantation, from embryonic day 4.5 to gastrulation. The conceptus at these stages has the shape of a cylinder and comprises two regions: the embryonic region distally and the extra-embryonic region proximally.

Extra-embryonic ectoderm (ExE)
This is a derivative of the trophectoderm, which underlies the inner cell mass (polar trophectoderm). Unlike cells of the ectoplacental cone (another trophectoderm derivative), which differentiate into giant cells, cells of the ExE retain stem-cell potential. The ExE contributes to the placenta.

Induction
A process whereby signaling from one group of cells affects the developmental program of the cells that receive the signal.

Primitive streak
A line of cells, initially visible at the posterior pole of the embryo, along which precursor cells of the mesoderm and the definitive endoderm ingress during gastrulation. It is essential for establishing the anterior–posterior axis and for segregating precursor cells of different organs. The primitive streak forms opposite to the anterior visceral endoderm.

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Mouse Genome Informatics
http://www.informatics.jax.org/
Integrated access to data concerning the genetics, genomics, and biology of the laboratory mouse

UNSW Embryology
http://embryology.med.unsw.edu.au/
An educational resource for learning concepts in embryologic development

11

SIGNALING CASCADES, GRADIENTS, AND GENE NETWORKS IN DORSAL/VENTRAL PATTERNING

GIRISH S. RATNAPARKHI and ALBERT J. COUREY

Department of Chemistry and Biochemistry, University of California at Los Angeles, Los Angeles, CA

INTRODUCTION

The specification of embryonic polarity resulting from the asymmetric distribution of gene products during oogenesis and early embryogenesis is the first step in metazoan development. Subsequent to the polarization of the embryo, coordinate axes are specified, thereby allowing each cell to sense its position within the organism and differentiate in a manner appropriate to this position. The number of axes is variable: the embryos of radially symmetric coelenterates possess just a single axis, whereas bilaterian embryos possess two axes: an anterior–posterior (AP) axis and a dorsal–ventral (DV) axis. This chapter introduces DV patterning in animals using the embryo of the fruit fly *Drosophila melanogaster* as an example. We describe the molecular events which are responsible for the specification and establishment of the DV axis from the oocyte to the blastoderm.

I. FORMATION OF THE DEVELOPMENTAL AXES AND SUBDIVISION OF THE EMBRYO INTO DEVELOPMENTAL DOMAINS

In Drosophila, polarity specification occurs during oogenesis (Roth and Schupbach, 1994; St. Johnston and Nusslein-Volhard, 1992; van Eeden and St. Johnston, 1999). After fertilization, activity gradients of transcription factors and signaling molecules form within the embryo and specify AP and DV coordinate axes (Morisato and Anderson, 1995; Nusslein-Volhard, 1991). The

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gradients are interpreted in terms of specific threshold concentrations (Driever and Nusslein-Volhard, 1988; Huang et al., 1997; Stathopoulos and Levine, 2002), and this leads to multiple discrete domains of gene expression along each axis. In many cases, these genes code for proteins that form additional gradients (Ferguson and Anderson, 1992a; Hulskamp et al., 1990; Srinivasan et al., 2002) that lead to the finer subdivision of the embryo.

Four gene networks define the polarity of the Drosophila embryo and establish the developmental axes. Three of these—the anterior, posterior, and the terminal systems—collaborate to define AP polarity and the AP axis, whereas the specification of DV polarity and the DV axis are the responsibility of the DV system (Anderson et al., 1985). Each system includes a set of maternally required genes, the mRNA products of which are synthesized in the nurse cells and transported into the oocyte late during oogenesis, and zygotically required genes, which are transcribed in the embryo a few hours after fertilization under the regulation of the maternal gene products.

After fertilization, the embryo undergoes 13 rapid mitotic cycles. These nuclear divisions are not accompanied by cytokinesis, and this results in the production of a syncytium containing more than 5000 nuclei. During the eighth nuclear cycle, the nuclei migrate to the periphery of the embryo; this leads to the formation of the syncytial blastoderm embryo by the start of the ninth cycle, in which the plasma membrane is lined with a single layer of nuclei. After the completion of the thirteenth nuclear cycle, cell membranes cleave in from the surface of the embryo, converting it into an ovoid-shaped monolayer epithelium called the *cellular blastoderm embryo*. The completion of cellularization is followed immediately by the cell movements that mark the onset of gastrulation, by which time DV axis formation and the initial subdivision of the embryo into broad developmental domains along its DV axis is complete.

During the syncytial blastoderm stage, the embryo is divided into three primary domains along the DV axis. These include a ventral domain, which will give rise during gastrulation to the mesoderm; a ventrolateral domain (the neurogenic ectoderm), which will give rise to the ventral epidermis and the central nervous system; and a dorsal/dorsolateral domain (the dorsal ectoderm), which will give rise to the dorsal epidermis and amnioserosa (a cell sheet that covers the dorsal-most surface of the embryo during much of embryogenesis but which is covered over by epidermal cells late during embryogenesis). The endoderm is derived from anterior and posterior regions after gastrulation is initiated by processes that are not described in this chapter.

The subdivision of the Drosophila embryo along its DV axis is directed by the opposing activity gradients of two factors: the transcription factor Dorsal (DL; Box 11.1), a member the rel transcription factor family and a homolog of vertebrate NF-κB, and the extracellular signaling protein Decapentaplegic (Dpp), a homolog of vertebrate bone morphogenetic proteins (BMPs) 4 and 2. The concentration of DL in the nucleus and therefore DL activity is maximal in the ventral regions of the syncytial blastoderm embryo, and it decreases dorsally. By contrast, Dpp signaling activity is maximal at the dorsal midline and decreases ventrally. Although the use of a rel family factor such as DL to regulate DV pattern formation may be unique to invertebrates, the use of Dpp/BMP2/4 in DV patterning is conserved in both invertebrates and vertebrates (Holley and Ferguson, 1997; Lall and Patel, 2001; Schmidt et al., 1995; see Chapter 12).

BOX 11.1 Gene/Protein Nomenclature

Drosophila biologists usually name genes according to the mutant phenotype (the visible effect of the mutation). For example, the *dorsal* (*dl*) gene is so named because embryos produced by mothers homozygous for a null allele of *dl* develop only dorsal structures (i.e., they are completely dorsalized). This finding suggests that *dl* is required for the formation of ventral structures, and, therefore, if *dl* had been named according to its function, it might have been called *ventral*. In general, the name of a Drosophila gene is italicized, whereas the name of the protein product is not italicized. The name of the protein is capitalized regardless of whether the gene name is capitalized. Hence, the *dpp* gene encodes the Dpp protein, and the *Tl* gene encodes the Tl protein. In the case of *dl*, the protein product is spelled in all capital letters (DL) to avoid confusion with the product of the *Delta* (*Dl*) gene.

TABLE 11.1 Drosophila Genes Discussed in this Chapter, Arranged in Alphabetical Order

Drosophila Gene (shorthand)	Structure and Function	Vertebrate Counterpart
brinker (brk)	Transcriptional repressor; expressed as a neuroectodermal stripe	
cactus (cact)	Binds to and sequesters DL in the cytoplasm	Iκ-B
cut (ct)	Transcriptional activator; homeodomain protein	CUT, CUTL1
decapentaplegic (dpp)	BMP ligand; forms DV activity gradient; cells receiving Dpp signals choose an ectodermal (nonneural) fate	BMP2/4, TGF-β superfamily
dorsal (dl)	Rel homology domain transcription factor; forms a DV nuclear gradient in Drosophila embryos	Rel family proteins, including NF-κB
Dri (dead ringer)/retained (retn)	Transcription factor binding AT-rich domain; part of a Gro recruiting platform	DRIL1
easter (ea)	Serine protease	Blood clotting proteases
Folded gastrulation (fog)	Secreted ligand; receptor not characterized	
gastrulation defective (gd)	Serine protease	Blood clotting proteases
groucho (gro)	Corepressor for DL	TLE family proteins
gurken (grk)	TGF-α–like protein; epidermal growth factor ligand	TGF-α family
intermediate neuroblasts defective (ind)	Homeobox gene	Gsh1, Gsh2
Krapfen (Kra)/ myd88 (myd88)	Adaptor protein; binds Tl and Tub	Myd88
medea (med)	Transcription factor downstream of Dpp; forms a complex with pMad	Smad4, Smad family
Mothers against dpp (Mad)	Transcription factor downstream of Dpp; phosphorylated in response to Dpp signaling	MADR1, Smad family
nudel (ndl)	Serine protease	

oelle (pll)	Serine/threonine kinase	IRAK
pipe (pip)	Heparan-sulfate-2-O-sulfotransferase	
pannier (pnr)	GATA transcription factor	
punt (pnt)	Transmembrane receptor kinase; receptor for Dpp/Scw	TGF-β receptor, type II
related to angiotensin-converting enzyme (race)	Zinc- and chloride-dependent peptidyl-dipeptidase activity	Angiotensin-I-converting enzyme
rhomboid (rho)	Intramembrane serine protease; targets Spitz	Rhomboid 1
saxophone (sax)	Transmembrane receptor kinase; receptor for Dpp/Scw	TGF-β receptor, type I
schnurri (shn)	Transcription factor	PRDII/MBPI/HIV-EP1
screw (scw)	BMP ligand; unrestricted expression	BMP2/4, TGF-β superfamily
Serpin27A (Spn27a)	Serine protease inhibitor; inhibits Ea	
short gastrulation (sog)	BMP signaling modulator; interferes with Dpp signaling and thus confers neuroectodermal fate	Chordin
single minded (sim)	bHLH-PAS protein	SIM
slalom (sll)	3′-phosphoadenosine 5′-phosphosulfate transporter	
snail (sn)	Transcriptionally represses genes that would confer neuroectodermal fate	SNAI1P
snake (snk)	Serine protease	Blood clotting proteases
spätzle (spz)	Tl ligand; proteolytic cleavage of Spz activates the ligand	
tailup (tup)	Transcription factor	LIM-homeodomain family
thickveins (tkv)	Transmembrane receptor kinase; receptor for Dpp/Scw	TGF-β receptor, type I
tinman (tin)	Transcription factor	Nkx-2.5/CSX1, NK2 family
Toll (Tl)	Transmembrane receptor; distributed evenly all over the embryonic membrane; binds Spz	Toll-interleukin–like (TIL) superfamily
tolloid (tld)	Metalloendopeptidase; cleaves Sog	BMP-1
Torpedo (top)	EGFR tyrosine kinase; transmembrane receptor	EGF family
tube (tub)	Functions as an adapter to recruit Pll	
tulip (tup)	3′-phosphoadenosine 5′-phosphosulfate transporter	
twist (twi)	bHLH protein; transcription factor	TWIST
twisted gastrulation (tsg)	Heparin binding; forms a complex with Dpp/Scw	Human connective tissue growth factor
vein (vn)	EGF ligand	Neuregulins
ventral nervous system defective (vnd)	Homeobox gene; Gro-dependent repressor	NK-2 family
windbeutel (wbl)	Endoplasmic reticulum protein	
zerknllt (zen)	Homeobox transcription factor	Hox class 3

II. FORMATION OF THE DL NUCLEAR CONCENTRATION GRADIENT

The formation of the DL nuclear concentration gradient (Ip et al., 1991; Roth et al., 1989; Rushlow et al., 1989; Steward et al., 1988) occurs as a result of two maternally encoded signaling cascades. In the first, a signal is relayed from the oocyte to the layer of follicle cells surrounding the oocyte and then back to the eggshell (Gonzalez-Reyes et al., 1995; Gonzalez-Reyes and St. Johnston, 1994). The net result is the deposition of a latent asymmetric signal (light gray arrows in Figure 11.1, B) in the perivitelline space (i.e., the space between the inner eggshell membrane [the vitelline membrane]) and the oocyte plasma membrane. After fertilization, this latent signal activates the second signaling cascade (Figure 11.2, A), which transduces the signal to the interior of the zygote and leads to the ventral-specific nuclear uptake of DL.

A. Interactions Between Follicle Cells and the Germ Line Define Dorsal–Ventral Polarity

Each Drosophila ovary is composed of approximately 16 ovarioles. Each ovariole is a string of egg chambers that emanates from an anterior germarium, which houses the germ line and somatic stem cells. As the egg chambers mature, they move posteriorly along the ovariole. Each egg chamber contains one posteriorly situated oocyte and 15 anteriorly situated polyploid nurse cells (Figure 11.1, A). These 16 cells are all descended from a single germ line progenitor as a result of four mitotic cycles. Cytokinesis during these mitotic cycles is incomplete, and, therefore, the 16 germ-line–derived cells are joined to one another via cytoplasmic connections called *ring canals*. During oogenesis, the nurse cells manufacture the massive amounts of protein and mRNA that will later pattern and sustain the embryo during early embryogenesis. The contents of the nurse cells are transported into the growing oocyte via the ring canals.

The 16 germ-line cells in each egg chamber are surrounded by a monolayer of follicle cells. These cells produce the eggshell, including the inner vitelline membrane and the outer chorion. In addition, as we will see, they receive signals from the oocyte, and, in response, the follicle cells send a polarizing signal back to the oocyte.

The primary event leading to DV polarity is the movement of the oocyte nucleus from an initial posterior position (dashed arrow in Figure 11.1, A) to an anterior–dorsal location, a movement that is dependent on a polarized, intact microtubule (MT) network within the oocyte. Reciprocal signaling between the follicle cells and the oocyte regulates this movement. Gurken (Grk), a transforming growth factor (TGF)-α–like ligand, is secreted by the oocyte, and it activates Torpedo (Top), the Drosophila epidermal growth factor receptor (EGFR), on the surface of follicle cells. An uncharacterized signal from the follicle cells back to the oocyte leads to the reorganization of the MT network, placing the minus end of the microtubules at the anterior end of the oocyte. Movement of the nucleus toward the minus end of the microtubules allows it to reach its final anterior–dorsal location (Gonzalez-Reyes et al., 1995; Gonzalez-Reyes and St. Johnston, 1994).

Through mechanisms that are not fully understood (Cáceres and Nilson, 2005), high levels of *grk* mRNA accumulate in a small arc between the nucleus and the plasma membrane (Figure 11.1, A). A second round of Grk signaling is then initiated from the oocyte to the overlying (dorsal) follicle cells, and

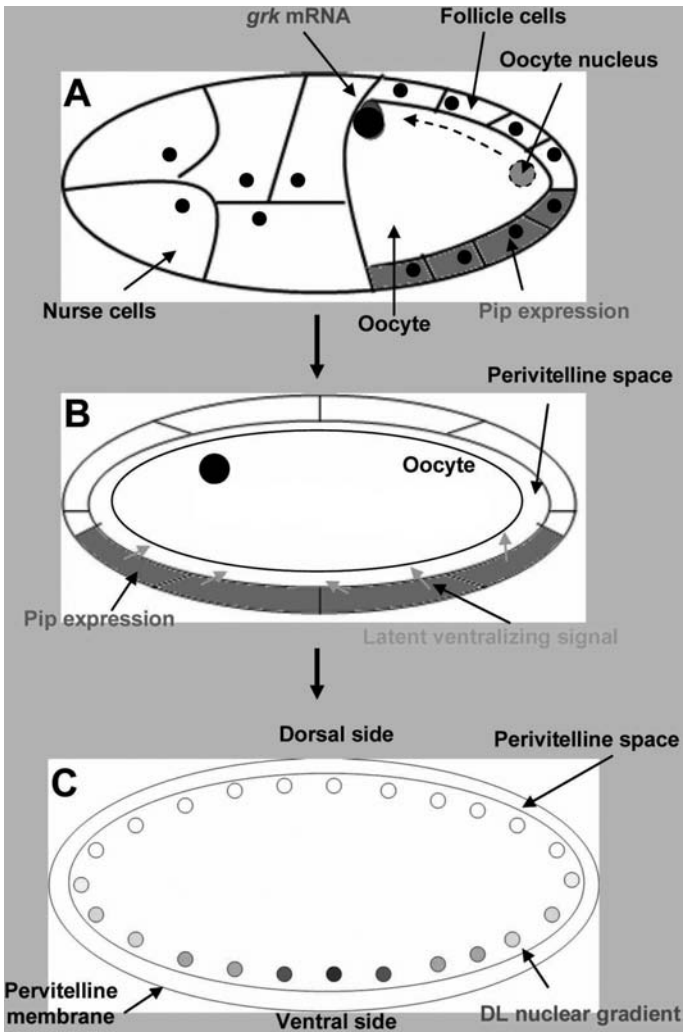

FIGURE 11.1 **From oocyte to embryo.** The establishment of the DV axis. **A**, Within an egg chamber, DV polarity in the oocyte results from asymmetric localization of the oocyte nucleus. The oocyte nucleus moves (shown by a *dashed arrow*) from an initial posterior location (*dashed margin*) to a final anterior location. The side to which the nucleus is closest upon its arrival at the anterior of the oocyte becomes the dorsal side of the future embryo. Specification of the dorsal side occurs when the oocyte nucleus produces *grk* mRNA, which accumulates in the region between the nucleus and the oocyte plasma membrane. **B**, Grk protein, which is secreted from the region that localizes *grk* mRNA, signals through the EGFR in the adjacent follicle cells leading to the repression of *pip* expression. *pip* expression is therefore restricted to the follicle cells on the ventral side of the future embryo (*shaded dark gray*). *pip* expression leads to the synthesis of a latent ventralizing signal (*light gray arrows*) that is deposited into the perivitelline space by the follicle cells before the egg shell is deposited. **C**, The dorsal gradient forms in the embryo a few hours after fertilization within the *pip* expression domain.

this results in a dorsal-to-ventral gradient of EGFR activation in the follicle cell epithelium. EGFR signaling inside the follicle cells then represses *pipe* (*pip*) expression. As a consequence of the EGFR activation gradient, *pip* expression is restricted to the follicle cells adjacent to the ventral side of the embryo (Figure 11.1, A and B). *pip* is expressed uniformly in the ventral

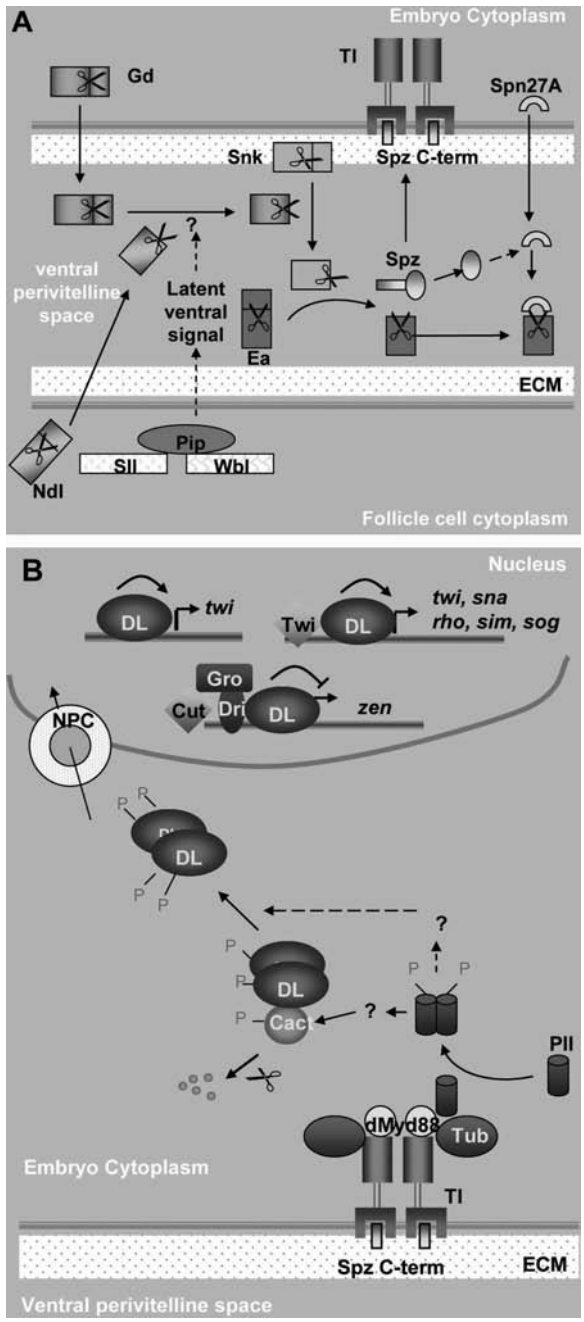

FIGURE 11.2 **Transduction of the latent ventralizing signal to the interior of the embryo leads to nuclear DL import. A,** After fertilization, the latent ventralizing signal triggers a cascade of proteolytic cleavages by serine proteases, which leads to the processing and activation of the Tl ligand Spz. The serine proteases are converted from an inactive state (shown as constrained scissors) to a proteolytically active state (scissors free to cleave). **B,** Asymmetric activation of the Tl receptor initiates a signal transduction pathway that leads to the degradation of Cact and the graded nuclear import of DL. The phosphorylation of both DL and Cact as a result of Tl signaling is critical for the transport of DL into the nucleus. After it is in the nucleus, DL activates and represses a number of genes that specify cell fate along the DV axis. (See color insert.)

40% of the follicle cells. The *pip*-expression domain has a sharp border that suggests that repression requires a certain threshold level of EGFR activity, which is only exceeded in the dorsal 60% of the egg chamber.

pip encodes a heparin sulphate 2-*O*-sulphotransferase that probably modifies the glycosaminoglycans (GAG) of the extracellular matrix (ECM) during their transit through the Golgi apparatus (Sen et al., 2000). GAGs are long, unbranched, sugar polymers that are attached to specific serine residues of proteins called *proteoglycans*. Support for the idea that Pip catalyzes GAG sulfation comes from studies showing that the synthesis of 3′-phosphoadenosine 5′-phosphosulfate (PAPS), the sulfate group donor for sulfotransferase reactions, and the import of PAPS into the Golgi apparatus are required for DV patterning. It is assumed that Pip modifies an unknown proteoglycan substrate and deposits it into the space outside of the oocyte before the vitelline membrane and chorion are secreted. Because *pip* mRNA is only expressed ventrally, the modified substrate is presumably restricted to the perivitelline space on the ventral side of the embryo, where it serves as a latent ventral signal (light gray arrows in Figure 11.1, B). As we will be described later, DL nuclear import after fertilization occurs in regions that abut the Pip-expressing follicle cells during oogenesis (Figure 11.1, B and C).

B. A Proteolytic Cascade in the Perivitelline Fluid Transduces the Ventral Signal from the Perivitelline Space to the Toll Ligand

The formation of the DL nuclear concentration gradient requires the activation of Toll (Tl), a receptor that is uniformly distributed throughout the plasma membrane of the syncytial embryo by its ligand, Spätzle (Spz). The production of activated Spz after fertilization requires a proteolytic cascade (Figure 11.2, A) in the ventral perivitelline space (LeMosy et al., 1999; Morisato and Anderson, 1994; Schneider et al., 1994; Smith and DeLotto, 1994). This cascade is initiated by the Pip-dependent latent signal in the pervitelline space, which is produced as described previously. The proteolytic cascade involves the successive activation of three germ-line–encoded serine proteases: Gastrulation defective (Gd), Snake (Snk), and Easter (Ea). The activation of Gd requires unknown Pip-modified substrates and Nudel (Nd), a serine protease that is secreted from follicle cells. Activated Gd then cleaves and activates Snk, which cleaves and activates Ea. Ea proteolytically processes and thereby activates Spz. The resulting C-terminal fragment of processed Spz (Schneider et al., 1994) binds and activates the Tl receptor (Figure 11.2, A and B).

As will be described later, the spatially restricted activation of the Tl receptor, which is uniformly distributed throughout the egg plasma membrane, results in the graded nuclear uptake of DL. It is not known how the uniform expression of *pip* in the ventral 40% of the egg chamber translates into the gradient of DL nuclear localization (Figure 11.1, A). It is possible that the Ea inhibitor Serpin27A (Spn27a) plays a role in this process (Hashimoto et al., 2003). This inhibitor is secreted from the oocyte, and it may be preferentially secreted from the dorsal side of the embryo as a result of the inhibition of secretion by Tl signaling. One possibility is that the ventral-to-dorsal diffusion of Ea together with the dorsal-to-ventral diffusion of Spn27A leads to a gradient of Ea activity (Chang and Morisato, 2002), which results in a gradient of processed Spz and therefore a gradient of Tl activity (Moussian and Roth, 2005; Stein et al., 1991).

C. Asymmetric Signaling by the Toll Receptor Leads to the Formation of a Dorsal Nuclear Concentration Gradient

Tl and its homologs in vertebrates (the Toll-like receptors) play conserved roles in the insect and vertebrate innate immune response (Anderson, 2000; Lemaitre et al., 1995). Thus, although the roll of Tl in DV pattern formation may not be conserved in vertebrates (as described later), studies of DV patterning in Drosophila and innate immunity have illuminated one another (Brennan and Anderson, 2004; Imler and Hoffmann, 2001).

Tl signaling in the syncytial embryo is required for the nuclear import of DL. DL, a transcription factor, is encoded by a uniformly distributed maternally supplied mRNA, and, therefore, the translation of the mRNA in the syncytial embryo results in the accumulation of uniformly distributed DL protein. Tl regulates DL nuclear uptake in two ways. First, the Tl signal leads to degradation of Cactus (Cact), an inhibitory factor and a homolog of vertebrate I-κB that binds DL and sequesters it in the cytoplasm (Belvin et al., 1995; Bergmann et al., 1996; Govind et al., 1993; Roth et al., 1991). Second, the Tl signal may act directly on DL to enhance its nuclear import. The asymmetric distribution of the Tl ligand results in a DL nuclear concentration gradient, with the highest levels of DL in the ventral nuclei, low levels of DL in the lateral nuclei, and little or no DL in the dorsal nuclei (Roth et al., 1989; Rushlow et al., 1989; Steward et al., 1988; Figure 11.3, A).

The Tl signaling pathway is thought to have the following features (Figure 11.2, B). The binding of activated Spz to the Tl receptor leads to the dimerization of Tl. Dimerized Tl recruits dMyD88 via a shared Toll and IL-1 receptor/resistance (TIR) motif. dMy88 complexes with Tube (Tub) via a DEATH domain motif that is present in both proteins (Feinstein et al., 1995). The Tl/dMyD88/Tub complex can now recruit the Pelle (Pll) kinase, which also contains a DEATH domain. The increase in the local concentration of these proteins near the Tl receptor leads to Pll autophosphorylation and also to the phosphorylation of Tl and Tub by Pll (Norris and Manley, 1996; Shen and Manley, 2002; Sun et al., 2002). This has two consequences. First, phosphorylated Pll is released from the complex; thus, signaling is rendered self-limiting. Second, the released phosphorylated Pll can now act on downstream components, which leads to the phosphorylation of Cact.

The question of whether Pll acts on Cact directly or indirectly is controversial. Support for direct action comes from *in vitro* assays, which indicate that Pll can directly phosphorylate Cact. In vertebrates, however, the DL homolog NF-κB is sometimes phosphorylated by the IκB kinase (IKK) complex, which includes the catalytic subunits IKKα and IKKβ. Support for the idea that similar kinases might regulate DL function comes from studies of innate immunity that show that IKKβ family kinase Ird5 is downstream of Pll and responsible for the phosphorylation of Cact (Lu et al., 2001). Ird5 mutations do not, however, result in major defects in DV pattern formation, which suggests that there may be multiple partially redundant kinases required for Cact phosphorylation.

Cact blocks DL nuclear uptake, perhaps by physically tethering DL in the cytoplasm (Figure 11.2, B). Phosphorylation of Cact leads to its dissociation from DL and also to its degradation (Govind et al., 1993; Reach et al., 1996; Roth et al., 1991), thereby allowing DL to interact with the nuclear importins, which escort DL into the nucleus via the nuclear pore complexes.

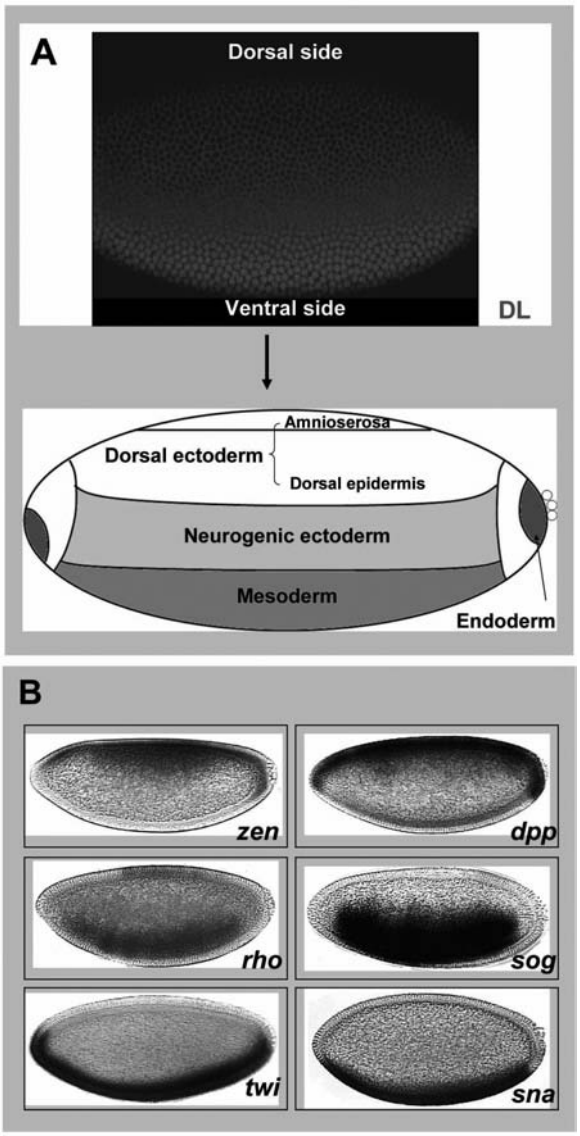

FIGURE 11.3 **Activation and repression by DL leads to the subdivision of the embryo into broad developmental domains. A**, The relationship between the DL nuclear gradient and the three primary developmental domains (dorsal ectoderm, neurogenic ectoderm, and mesoderm) at the cellular blastoderm stage. The DL gradient is visualized using an antibody that recognizes the DL Rel homology domain. **B**, A subset of genes that are activated *(twi, sna, sog, rho)* and repressed *(dpp, zen)* by DL. DL and DL target genes specify cell fate in the ventral and lateral domains of the embryo, whereas Dpp signaling specifies cell fate in dorsal and dorsolateral domains of the embryo. The mRNA for each gene is visualized by hybridization to labeled RNA probes.

In the case of the I-κB, the vertebrate homolog of Cact, degradation depends on the ubiquitin/proteasome pathway. Phosphorylation of I-κB by IKK at two specific serine residues leads to recognition and polyubiquitylation by a ubiquitin ligase containing the F-box protein β-TrCP. The polyubiquitylated protein is then recognized and degraded by the 26S proteasome (Spencer et al., 1999). The phosphorylation sites in I-κB are not conserved in Cact.

Nonetheless, embryos deficient in Slimb, which is the Drosophila homolog of β-TrCP, exhibit DV patterning defects that suggest that Cact degradation occurs by a pathway that is similar to the I-κB degradation pathway.

DL itself is phosphorylated (Drier et al., 1999) in a manner that is dependent on activated Tl, and this appears to be required for nuclear import (Figure 11.2, B). Further evidence for the physiologic significance of DL phosphorylation comes from studies of a mutant form of DL that is unable to bind Cact. Nuclear entry of this form of DL is not completely unregulated as one might expect if all Tl signaling went through Cact. Instead, although the DL gradient is extended, more nuclear uptake occurs ventrally than dorsally, and this nuclear uptake is dependent on the genes that are responsible for Spz processing (Drier et al., 2000).

III. SUBDIVISION OF THE EMBRYO INTO MULTIPLE DEVELOPMENTAL DOMAINS BY THE DORSAL NUCLEAR CONCENTRATION GRADIENT

As discussed previously, the blastoderm embryo contains three DV developmental domains: the mesoderm, the neurogenic ectoderm, and the dorsal ectoderm (Figure 11.3, A). DL establishes these domains by functioning as both a transcriptional activator and a repressor to direct the spatially restricted expression of zygotically active DV patterning genes (reviewed in Stathopoulos and Levine, 2002). In general, DL activates the genes that are required for the mesodermal and neurogenic ectodermal fates (Figure 11.3, A).

A. Dorsal Activates a Number of Genes in the Blastoderm Embryo

On the basis of a genomic promoter/enhancer sequence analysis, DL is believed to modulate the expression of 50 to 60 genes in the blastoderm embryo (Markstein et al., 2002; Stathopoulos et al., 2002). Of these, about 30 have been experimentally verified as DL targets. DL regulates these target genes via enhancer or silencer elements containing critical DL binding sites. DL-dependent enhancers are called *ventral activation regions* (VARs), whereas DL-dependent silencers are called *ventral repression regions* (VRRs; Courey and Jia, 2001). Whether or not DL activates or represses any given target gene and the threshold concentration of DL required for this activation or repression depends on the quality and context of the binding sites (Ip et al., 1991; Jiang et al., 1991; Pan and Courey, 1992).

Genes such as twist *(twi)* and snail *(sna)*, which encode mesodermal determinants, are only activated by the high concentrations of DL present in the ventral mesodermal anlage, whereas genes such as short gastrulation *(sog)* and rhomboid *(rho)*, which are required for neurogenic ectodermal development, can be activated at lower DL concentrations (Figure 11.3, B). *sog*, for example, is initially activated throughout the mesodermal and neurogenic ectodermal anlagen (the ventral 60% of the blastoderm embryo; Stathopoulos and Levine, 2002). These neurogenic ectodermal genes are, however, very rapidly repressed by Sna in the presumptive mesoderm. DL functions as a repressor of genes such as *dpp, zerknllt (zen)*, and *tolloid (tld)*, which are required for the dorsal ectodermal fate; in this way, their expression is restricted to the dorsal-most 40% of the embryo (i.e., the region lacking nuclear DL; Figure 11.3, B; Ip et al., 1992; St. Johnston and Gelbart, 1987; Thisse and Thisse, 1992).

B. Dorsal Binding Affinity and Synergistic Interactions Determine the Borders of the Expression Domains

Binding sites that closely match the DL consensus recognition element are of high affinity and therefore interact with DL at lower concentrations. Thus, high-affinity binding sites direct activation or repression in a broader ventral domain than do low-affinity DL binding sites (Jiang and Levine, 1993). For example, the *twi* gene encodes a mesodermal determinant that is only expressed at high levels in the ventral 20% of the blastoderm embryo, where DL concentration is highest. Accordingly, the two VARs in Twi contain only low-affinity DL binding sites that match the consensus very poorly. By contrast, the *zen* gene encodes a dorsal ectodermal determinant that must be repressed by DL throughout the mesodermal and neurogenic ectodermal anlagen. Accordingly, the *zen* VRR contains several consensus and therefore high-affinity DL binding sites (Stathopoulos and Levine, 2002).

The size of a domain of DL-mediated activation can be influenced by synergistic interactions between DL and other activators that are bound to nearby sites (Jiang and Levine, 1993). Expanded domains of activation of certain neurogenic ectodermal genes such as *rho* depend on binding sites for basic-helix-loop-helix (bHLH) transcription factors close to the DL binding sites. bHLH factors, such as Twi and Daughterless (Da), bind to these sites and synergize with DL, thereby allowing for activation in regions of lower DL concentration (Jiang and Levine, 1993). In general, the molecular mechanisms behind this synergy have not been well defined.

C. Dorsal Can Also Function as a Groucho-Dependent Repressor

Although DL is, by default, a transcriptional activator, it also actively represses the expression of some of the genes required for the formation and patterning of the dorsal ectoderm, including *dpp, zen,* and *tld* (St. Johnston and Gelbart, 1987). Repression of these targets requires DL-mediated recruitment of the corepressor protein Groucho (Gro) to the VRRs in these genes (Figure 11.2, B; Dubnicoff et al., 1997). The affinity of DL for Gro appears to be quite low, thereby explaining why DL is normally an activator rather than a repressor. The repression of targets such as *zen* and *dpp* requires additional repressor proteins that bind to nearby sites in the VRRs and that apparently assist DL in the recruitment of Gro. This has been best studied in the case of the *zen* gene, which contains a VRR in the 5′ flanking region. The *zen* VRR contains three high-affinity DL binding sites as well as three evolutionarily conserved adenine–thymine (AT)-rich elements. These AT-rich elements serve as binding sites for the sequence-specific transcription factor Dead ringer (Dri); biochemical experiments show that DL and Dri bound to adjacent sites in DNA can cooperatively recruit Gro to the DNA. This has led to the conclusion that DL and Dri serve to nucleate the formation of a Gro-containing multiprotein DNA-bound complex (a "repressosome") that is required for DL-mediated silencing (Valentine et al., 1998). Support for this conclusion comes from experiments demonstrating that the mutagenesis of the AT-rich elements or the elimination of Dri from the early embryo converts the zen VRR to a VAR (Jiang et al., 1993; Valentine et al., 1998). Furthermore, the activity of the VRR is critically dependent on the spacing between the DL and Dri binding sites (Jiang et al., 1993). Thus, when the spacing between one of the DLs and one of the AT-rich sites was increased by a

nonintegral multiple of the helical repeat, repression was lost that was consistent with the idea that DL and Dri must be aligned on the same face of the helix to allow for efficient Gro recruitment.

IV. DECAPENTAPLEGIC/SHORT GASTRULATION ACTIVITY GRADIENTS ARE RESPONSIBLE FOR FURTHER PATTERNING OF DORSAL–VENTRAL AXIS

A. Repression and Activation by Dorsal Establishes the Polarity of the Decapentaplegic/Short Gastrulation Pattern-Forming System

As discussed previously, patterning of the Drosophila DV axis requires a DL nuclear concentration gradient (Figure 11.3, A). This DL gradient serves two broad purposes. First, different threshold concentrations of DL activate the genes that are required for the establishment and development of the ventral mesodermal domain and the ventrolateral neuroectodermal domain, including *sna, twi, rho,* and so on. Second, the DL gradient activates and represses the genes that encode some of the components of the Dpp/Sog morphogen system, which is required for the subdivision of the dorsal ectodermal domain. Although the DL system may not be required for DV patterning in vertebrate embryos, we will see that the Dpp/Sog system plays a role in patterning the vertebrate DV axis that is homologous to its role in Drosophila embryogenesis.

As mentioned previously, Dpp is an extracellular signaling molecule with a high degree of similarity to vertebrate BMPs 2 and 4, and it is a member of the TGF-β superfamily (Ferguson and Anderson, 1992b). Sog (Francois et al., 1994; Holley et al., 1996; Schmidt et al., 1995) is also a secreted protein, and it is related to vertebrate Chordin. *dpp* transcription is activated by uniformly distributed activators, but it is repressed by DL, which restricts *dpp* transcription (Ray et al., 1991) to the dorsal ectodermal anlage (roughly the dorsal-most 40% of the embryo). *sog* transcription is activated by DL throughout the mesodermal and neurogenic ectodermal anlagen (roughly the ventral-most 60% of the embryo). Thus, because DL activates *sog* but represses *dpp,* the transcripts of these two genes accumulate in nonoverlapping but abutting domains; *dpp* is transcribed where DL is absent, whereas *sog* is transcribed where DL is present (Figures 11.3, B, and 11.4). As we will see, despite their nonoverlapping domains of expression, Sog works with Dpp to subdivide the dorsal ectoderm into amnioserosa and dorsal epidermis (reviewed in Ashe, 2005).

B. The Bone Morphogenetic Protein Signal Transduction System

Patterning of the dorsal ectoderm requires, in addition to Dpp, a second BMP family ligand, Screw (Scw; Arora et al., 1994). Like all members of the TGF-β family superfamily, Dpp and Scw function as dimers (either homodimers or heterodimers). The Dpp/Scw heterodimer is thought to be a much more potent signaling agent than either homodimer. Each BMP receptor contains type I and type II subunits (probably two molecules of each), and each subunit possesses serine/threonine kinase activity. The Drosophila genome encodes a single type II kinase called Punt (Pnt) that is common to all of the Drosophila BMP receptors and two type I kinases called Thickveins (Thv) and Saxophone (Sax), which are required for signaling by Dpp and Scw, respectively (Nguyen et al., 1998; Ruberte et al., 1995).

Ligands are thought to recruit the constitutively active type II kinases to the type I kinases, thereby allowing for the phosphorylation and activation of the type I kinases. This, in turn, leads to the phosphorylation of the Smad family transcription factor Mad, which binds to another Smad family factor, Medea (Med), enters the nucleus, and activates and represses downstream genes that regulate developmental fate.

With the recent development of tagged forms of Dpp (Shimmi et al., 2005; Wang and Ferguson, 2005), it has become possible to visualize the distribution of Dpp protein. During early cellular blastoderm formation, Dpp protein is distributed in a broad dorsal domain that reflects the broad distribution of the *dpp* transcript. However, as cellularization proceeds, a dramatic redistribution occurs: Dpp comes to be localized in a step gradient, with high concentrations in a stripe along the dorsal midline (in the presumptive amnioserosa) and lower concentrations in dorsolateral regions (the presumptive dorsal epidermis) (Figure 11.4, A; Ashe et al., 2000). This asymmetric distribution of Dpp is what drives the subdivision of the dorsal ectoderm into amnioserosa and dorsal epidermis (Figures 11.3, A, and 11.4, A).

C. Diffusion of Short Gastrulation Toward the Dorsal Midline Leads to the Formation of a Decapentaplegic Activity Gradient

The redistribution of Dpp is thought to depend on Sog (Figure 11.4, A; Ashe and Levine, 1999; Francois et al., 1994; Marques et al., 1997). Sog forms a gradient with high concentrations at the dorsal edge of *sog* expression (on the lateral side of the embryo) and lower concentrations toward the dorsal midline (Srinivasan et al., 2002). Genetic analysis suggests that Sog plays both negative and positive roles in Dpp regulation (Araujo and Bier, 2000; Biehs et al., 1996; Decotto and Ferguson, 2001; Francois et al., 1994). Sog binds to both Dpp homodimers and Dpp/Scw heterodimers in complexes that also contain the product of the *twisted gastrulation* (*tsg*) gene. When present in this complex, Dpp and Scw are unable to bind their receptors (Figure 11.4, B). This prevents them from interfering with the normal developmental program in the neuroectodermal and mesodermal domains. At the same time, Sog facilitates the diffusion of these ligands through the perivitelline space (the red arrows in Figures 11.4, A and B) to the dorsal midline, resulting in the zone of high Dpp concentration at the dorsal midline (Ashe, 2005; Shimmi et al., 2005; Wang and Ferguson, 2005).

How can the diffusion of Sog generate a zone of concentrated Dpp around the dorsal midline? Key to this process is the protease encoded by the *tolloid (tld)* gene and expressed on the dorsal side of the embryo (the scissors in Figure 11.4, B). This protease cleaves Sog. Importantly, however, the protease does not cleave free Sog but only Sog in association with the BMP ligands. It is most active on complexes containing the Dpp/Scw heterodimer. This Tld cleavage of Sog liberates the Dpp/Scw heterodimer from the complex, potentially allowing it to bind its receptor (Shimmi et al., 2005; Wang and Ferguson, 2005) and signal on the dorsal side or to bind to free Sog. Around the dorsal midline, the concentration of Sog is at a minimum, and it is too low to allow for the efficient rebinding of Sog to Dpp/Scw after Tld-mediated Sog degradation. Therefore, near the dorsal midline, the consequence of Sog degradation is the immobilization of Dpp and Scw by binding to their receptors. The net result is the accumulation of high concentrations of the Dpp/Scw heterodimer around the dorsal

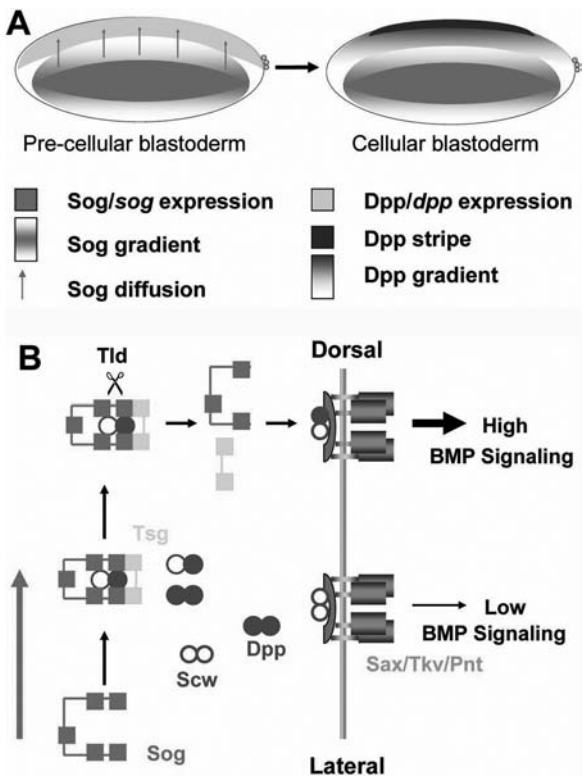

FIGURE 11.4 **Formation of a DV Dpp gradient. A**, *sog* is a DL target gene that is expressed in a broad lateral stripe in the blastoderm embryo. Sog is secreted into the extracellular space and diffuses away from the domain of *sog* expression, thus forming Sog gradients both ventral and dorsal to the zone of *sog* expression. In the dorsal region, the interaction of Sog with Dpp leads to the conversion of a homogenous Dpp expression domain in the dorsal 40% of the precellular embryo into an extracellular Dpp activity gradient in the cellular blastoderm embryo. Ultimately, extracellular Dpp comes to be distributed in a step gradient, with the highest concentrations at the dorsal midline and lower concentrations laterally. **B**, Sog binds to Dpp/Scw heterodimers in a complex that also includes Tsg and that facilitates the diffusion of Dpp by blocking binding to the transmembrane receptor composed of Sax, Tkv, and Pnt gene products. At the dorsal midline, the degradation of Sog by Tld liberates the Dpp/Scw heterodimer. As a result of the low concentrations of intact Sog at the dorsal midline, the Dpp/Scw heterodimer does not rebind Sog but instead binds the receptor. This leads to the accumulation of Dpp/Scw at the dorsal midline, which results in the formation of the Dpp step gradient. (See color insert.)

midline (Figure 11.4, B). Although it is possible that this sharp-step gradient of Dpp localization and signaling is exclusively the result of the Sog gradient, it seems more likely that the sharpening of the Dpp stripe requires positive autoregulation by the Dpp signaling pathway (Wang and Ferguson, 2005).

D. Redundancy in the Bone Morphogenetic Protein Signaling System

As described previously, the activation of Sog by DL on the ventral side of the embryo is a key step in the establishment of positional information by the BMP morphogen system, because the diffusion of Sog from a ventral source leads to the formation of a gradient of Dpp signaling activity. At the same time, DL also represses both *dpp* and *tld* ventrally, thereby producing additional spatial information that is at least partially redundant with the spatial

information provided by the ventral specific activation of *sog*. A DL variant which can activate but not repress transcription patterns the DV axis almost normally (Ratnaparkhi et al., 2006). This indicates redundancy in repression of Dpp/*dpp* signaling by indicating that DL-mediated repression is not essential for specification of the DV axis. DL activation (of sog and brk) by itself appears to be necessary and sufficient to pattern the DV axis. DL is one of the few rel family transcription factors known to actively repress transcription; most other members of this family appear to be dedicated activators. DL may have evolved the ability to repress *dpp* and *tld* expression after the vertebrate/invertebrate evolutionary split.

Additional redundancy in the BMP patterning system is found downstream of receptor activation, and it involves the action of the transcriptional repressor Brinker (Brk). The *brk* gene is activated by DL, and its expression is therefore limited to ventral regions where Dpp signaling is low or absent. Brk represses some of the same targets that are activated by pMad/Med. As a result, BMP targets are both activated dorsally by pMad/Med and repressed ventrally by Brk, which redundantly ensures that these targets will only be expressed in dorsal regions (Affolter et al., 2001; Jazwinska et al., 1999; Minami et al., 1999).

The redundancy of this spatial information is also illustrated by experiments in which tagged forms of Dpp are ectopically expressed along the entire DV axis of the embryo. In these experiments, most of the tagged Dpp still manages to find its way to the dorsal midline of the embryo. Thus, the asymmetric expression of the *dpp* gene appears to be dispensable for the ultimate asymmetric distribution of the Dpp protein as long as the Dpp inhibitor Sog is asymmetrically distributed (Shimmi and O'Connor, 2003; Shimmi et al., 2005; Srinivasan et al., 2002).

Such redundant mechanisms are being increasingly found in other developmental pathways (Barolo and Posakony, 2002). Although the exact reason for the multiple forms of redundancy in the BMP system is not clear, it is likely that it helps to render the pattern-forming system robust in the face of variations in environmental conditions that might lead to minor perturbations of the Sog gradient.

V. THE DORSAL–VENTRAL REGULATORY NETWORK

The preceding sections of this chapter describe a complex gene regulatory network (GRN) involving the DL and Dpp/Sog gradients, the genes that direct the formation of these gradients, and the genes that are targeted by these gradients (Figure 11.5; reviewed in Levine and Davidson, 2005). The regulatory interactions that have been discussed have all been illuminated by traditional genetic and biochemical approaches. However, the use of bioinformatic methods to analyze the Drosophila genome, microarray methods, and the systematic examination of gene-expression patterns are leading to the discovery of additional members of the DV GRN, which is now known to include 60 genes (Figure 11.5; Levine and Davidson, 2005; Stathopoulos et al., 2002), about 40 of which are expressed at the cellular blastoderm stage.

Features of gene regulation and pattern formation that were not obvious from the analysis of single genes can be appreciated at the level of the network. For example, by identifying genes with novel expression patterns, it may be possible to illuminate further the combinatorial interactions responsible

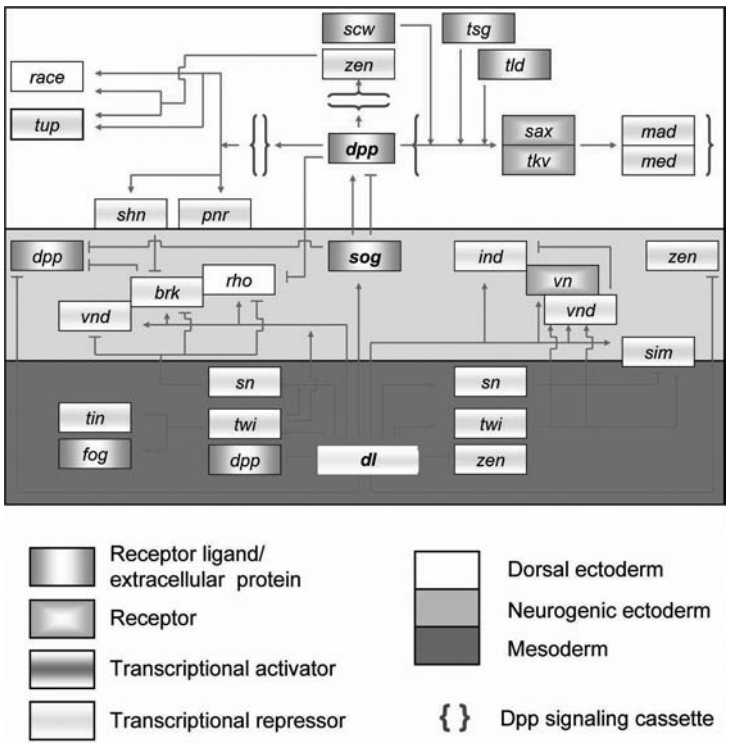

FIGURE 11.5 **A network of gene regulation in Drosophila DV patterning.** The DL and Dpp activity gradients lead to the spatially restricted activation and repression of a number of zygotically active DV patterning genes. Most of these genes are transcription factors or signaling molecules that are all linked in a complex gene regulatory network (Levine and Davidson, 2005). Each developmental domain (dorsal ectoderm, neurogenic ectoderm, or mesoderm) expresses a set of DL or Dpp target genes that initially mark and later specify the domain. Arrows (→) indicate the activation of a gene; bars (—) indicate the repression of a gene. DL is bifunctional and activates some genes (e.g., *twi*, *sna*, *sog*) while repressing others (e.g., *dpp, zen, tld*) when it is imported to the nucleus. DL blocks Dpp (BMP) signaling in the mesoderm and the neurogenic ectoderm by the transcriptional repression of *dpp* (Figure 11.3, *B*). This repression is supplemented by at least two additional pathways that inhibit Dpp signaling. These include the inhibition of the interaction between Dpp and its receptor by Sog and the repression of Dpp target genes by Brk. Dorsal ectoderm forms in the domain where Dpp signaling occurs, whereas neurogenic ectoderm forms in lateral regions that lack Dpp signaling and that also do not express Sna. Mesoderm forms in the ventral domain, where DL, Twi, and Sna are expressed together. (See color insert.)

for subdivision of the DV axis. In addition, the elucidation of gene networks that regulate the same process (e.g., germ layer specification) in diverse species will provide insights into mechanisms of molecular evolution.

VI. COMPARISON OF DORSAL–VENTRAL PATTERING IN DROSOPHILA AND VERTEBRATES

A. Tl Signaling May Contribute to the Specification of the Vertebrate Dorsal–Ventral Axis

The Tl signaling pathway has at least two functions in the life of the fruit fly. In addition to its role in embryonic DV patterning, this pathway is also required

for the Drosophila immune response (Anderson, 2000; Imler and Hoffmann, 2001). In insects, immunity means innate immunity, because insects lack the systems required for adaptive immunity (e.g., B and T lymphocytes). The innate immune response is triggered by the recognition of features that are common to many pathogens, such as microbial cell-wall–derived lipopolysaccharides, peptidoglycans, and lipoproteins. These molecules trigger the production of antimicrobial peptides, which lyse the invading microbes (the humoral response), and the activation of macrophages, which engulf the microbes (the cellular response). Both of these responses are partially dependent on the Tl receptor along with many other components of the Tl pathway. Although DV pattern formation is directed by a single rel family protein, namely DL, the innate immune response in Drosophila is directed by DL and two additional rel family proteins called DL-like immunity factor (Dif) and Relish.

Vertebrate genomes encode a signaling pathway that is highly homologous to the Drosophila Tl pathway. Like the insect Tl pathway, the vertebrate pathway has critical roles in innate immunity. For example, vertebrate genomes encode multiple Tl-like receptors that recognize conserved microbial features and then trigger cellular and humoral innate immune responses. These signals are, in many cases, transduced by phosphorylation cascades that lead to the destruction of the Cact homolog I-κB and the consequent nuclear import of rel family transcription factors such as NF-κB.

Although the roll of the Tl pathway in innate immunity is clearly conserved in both vertebrates and invertebrates, the evidence to show a role for Tl signaling in vertebrate DV axis formation is not definitive. *Drosophila* Spz and Tl can rescue DV pattern formation in Xenopus embryos after ultraviolet irradiation (a treatment that abolishes DV pattern; Armstrong et al., 1998). Also, the expression of a dominant negative form of Xenopus MyD88 blocks Tl receptor activity, inhibits axis formation, and reduces the expression of pivotal organizer genes (Prothmann et al., 2000). Thus, the Tl pathway may have an ancient role in DV patterning that predates the evolutionary split between vertebrates and invertebrates. However, in the absence of loss-of-function genetic evidence, it is possible that the only broadly conserved role for the Tl pathway is in innate immunity and that this pathway has been co-opted for DV pattern formation during relatively recent insect evolution.

B. Decapentaplegic/Short Gastrulation Orthologues Pattern Both the Invertebrate and Vertebrate Dorsal–Ventral Axes

The DV axis is reversed in vertebrates as compared with invertebrates (Arendt and Nubler-Jung, 1994; Gerhart, 2000; Holley et al., 1995). In invertebrates, for example, the nerve chord is a ventral structure, whereas the heart is a dorsal structure. In vertebrates, however, the nerve chord is dorsal, and the heart is ventral. As described later, recent studies showing that DV patterning in vertebrate embryos depends on the BMP morphogen system suggest that this difference may be superficial in nature (Gerhart, 2000).

Classic studies show that a piece of tissue (the Spemann organizer) from the dorsal lip of the amphibian blastopore can induce dorsal structures in the ventral mesoderm and in embryos that have been ventralized by ultraviolet irradiation. Recent experimentation has shown that Chordin, the vertebrate ortholog of Sog, is partially responsible for the dorsalizing activity of the Spemann organizer and that it functions by antagonizing BMP4 (a Dpp

homolog; Holley et al., 1995; Schmidt et al., 1995). Thus, although Sog/Chordin expression defines the ventral pole of the Drosophila embryo, it also defines the dorsal pole of the frog embryo. Conversely, although Dpp/BMP4 expression defines the dorsal pole in Drosophila, it also defines the ventral pole in frogs. Therefore, the apparent reversal of the body plan between vertebrates and invertebrates is likely an artifact of the way that early anatomists decided to define DV polarity in these two groups of animals.

Definitive proof that a BMP morphogen system organizes the vertebrate DV axis comes from loss-of-function genetic analysis carried out in zebrafish. Mutations in the genes encoding the zebrafish orthologs of BMP ligands and receptors result in the dorsalization of the embryo, whereas a mutation in the gene encoding the zebrafish ortholog of Chordin leads to the ventralization of the embryo. Furthermore, the analysis of mutations in the gene encoding the zebrafish counterpart of Tld suggests that, like Drosophila Tld, zebrafish Tld potentiates BMP signaling, presumably by degrading Chordin (Yamamoto and Oelgeschlager, 2004).

C. Drosophila as a Model Organism for Studying Development and Disease

Genome sequences of divergent animals, including *Drosophila melanogaster* (fruit fly), *Caenorhabditis elegans* (round worm), *Danio rerio* (zebrafish), *Mus musculus* (mouse), and *Homo sapiens* (man), confirm that invertebrates and mammals share many common genes (Box 11.2). Genetic and biochemical analyses also show that GRNs are highly conserved across species. Frequently, homologous GRNs have homologous functions in different species, although sometimes a GRN evolves a new function that is unique to one species or group of species. For example, the DL/NF-κB network has homologous functions in innate immunity in vertebrates and invertebrates, whereas the function of this network in DV pattern formation may be unique to invertebrates. Nonetheless, the use of genetic approaches to characterize the genes

BOX 11.2 Drosophila in the Study of Early Embryonic Development

Drosophila has a number of advantages that make it ideal for studying development by genetic approaches, including the following: a short generation time (10 days); a simple genome (one-twentieth the size of the human genome); and the availability of vectors for introducing modified genes and expressing them in a temporally and spatially regulated manner. Studies of Drosophila embryogenesis were pioneered by Christiane Nusslein-Volhard and Eric Wieschaus, who carried out extensive screens for zygotic and maternal mutations that disrupt embryogenesis. Many of these mutations resulted in axial (dorsal–ventral, anterior–posterior) patterning defects (Nusslein-Volhard, 1991; Nusslein-Volhard and Wieschaus, 1980). Genetic techniques were used to show that one set of genes defined in these screens—the "dorsal group," which includes *dl*—encoded the components of a pathway that directed the patterning of the dorsal–ventral axis (Ray et al., 1991). These studies led to prediction of the DL protein gradient in 1979, which was 10 years before the gradient was actually visualized by three independent groups (Roth et al., 1989; Rushlow et al., 1989; Steward et al., 1988). Nusslein-Volhard and Wieschaus were honored with the 1995 Nobel Prize for Physiology and Medicine, along with Edward B. Lewis, for their discoveries concerning the genetic control of early embryonic development.

BOX 11.3 Of Flies and Men

The sequences of the fly and human genomes reveal a strikingly high degree of similarity between these organisms (Adams et al., 2000; Venter et al., 2001). The fly genome contains approximately 13,000 genes as compared with the approximately 25,000 genes in humans. Current estimates indicate that at least 30% of the known 2300 human disease genes have well-conserved counterparts in flies (Bier, 2005). The human disease genes include genes that are involved in cancer as well as those involved in neurologic, cardiovascular, endocrine, and metabolic diseases. Many of the genes that play important roles in mammalian homeostasis were in fact initially discovered and characterized in flies. Because of the ease with which genetic screens can be carried out in Drosophila, flies continue to be one of the premier systems for discovering new gene functions. Such studies greatly aid our understanding of human development. Drosophila is also being increasingly used as a transgenic model for studying the genes that cause human disease.

that pattern the Drosophila DV axis has greatly informed our understanding of innate immunity in all organisms. Thus, studies of Drosophila DV patterning are relevant to an understanding of human immunodeficiency disorders (Box 11.3).

Similarly, the Dpp/BMP network has conserved functions in both vertebrate and insect DV patterning, but it also has many roles that are unique to insects, including roles in wing and leg development during metamorphosis. The powerful genetic approaches available to study insect metamorphosis can therefore be used to great advantage to decipher the functional interactions that comprise this network. The BMP pathway also has roles that are unique to vertebrates, such as roles in bone morphogenesis. For example, a gain-of-function mutation in a gene encoding the human homolog of Thickveins (the type I Dpp receptor) is responsible for fibrodysplasia ossificans progressiva, a devastating disease that results from massive bone overgrowth (Shore et al., 2006). Studies of Drosophila embryogenesis and metamorphosis have greatly increased our understanding of the molecular basis for this disease (Boxes 11.2 and 11.3).

SUMMARY

- Grk, a TGF-α family protein, is secreted by the Drosophila oocyte, and it signals via the EGFR in the adjacent follicle cells. This signaling specifies DV polarity in the follicle cell epithelium, which in turn deposits a latent asymmetric cue in the ventral perivitelline space.
- After fertilization, the activation of Tl by the ventral cue results in Tl signaling on the ventral side of the embryo and leads to the formation of a ventral-to-dorsal nuclear concentration gradient of the protein DL.
- The DL protein is a transcription factor that serves as a morphogen to direct cell fate as a function of position along the DV axis. It does so by regulating approximately 50 genes in the blastoderm embryo. Different genes are activated or repressed at different threshold DL concentrations, and this results in multiple domains of gene expression. The action of this complex gene network specifies and further subdivides the presumptive mesoderm, neurogenic ectoderm, and dorsal ectoderm.

- Two of the genes regulated by DL, Dpp and Sog, are critical components of a second morphogen system required for the specification of cell fate along the DV axis. Sog both enhances and inhibits Dpp signaling activity to subdivide the dorsal ectoderm into epidermis and amnioserosa.
- Loss- and gain-of-function genetic analyses carried out in flies, frogs, and fish demonstrate that the role of the Dpp/Sog morphogen system in patterning the DV axis is conserved in both invertebrates and vertebrates.

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INTERNET RESOURCES FOR EXPLORING DROSOPHILA DEVELOPMENT

Flybase
www.flybase.org—A database of the Drosophila genome.
Flybase: Drosophila anatomy and images
http://flybase.org/anatomy/.

Flybase: The interactive fly
http://flybase.bio.indiana.edu/allied-data/lk/interactive-fly/aimain/1aahome.htm.

Flymove
http://flymove.uni-muenster.de/—Images, movies and interactive Shockwave files of Drosophila development.

The WWW Virtual Library: Drosophila
http://www.ceolas.org/fly/—Internet resources for research on the fruit fly.

Berkeley Drosophila Genome Project
http://www.fruitfly.org/EST/index.shtml.

Berkeley Drosophila Genome Project: Patterns of gene expression in Drosophila embryogenesis
http://www.fruitfly.org/cgi-bin/ex/insitu.pl.

FlyView: A Drosophila Image Database
http://flyview.uni-muenster.de/.

Curagen
http://portal.curagen.com/cgi-bin/interaction/flyHome.pl—Drosophila interaction database.

Flymine
http://www.flymine.org/—An integrated database for Drosophila and Anopheles genomics.

Homophila
http://superfly.ucsd.edu/homophila/—Human disease to *Drosophila* gene database.

PubMed:
www.ncbi.nlm.nih.gov/entrez/query.fcgi?DB=pubmed—The National Center for Biotechnology Information's digital online archive for journal literature using the *Entrez* integrated, text-based, search and retrieval system.

Christiane Nusslein-Volhard - Autobiography
http://nobelprize.org/medicine/laureates/1995/nusslein-volhard-autobio.html.

12 EARLY DEVELOPMENT OF EPIDERMIS AND NEURAL TISSUE

KEIJI ITOH and SERGEI Y. SOKOL

Brookdale Department of Molecular, Cell and Developmental Biology, Mount Sinai School of Medicine, New York, NY

INTRODUCTION

The generation of the three germ layers—the ectoderm, the mesoderm, and the endoderm—is among the earliest and the most fundamental processes underlying animal development. The main derivatives of ectoderm (the outer germ layer) are the central and the peripheral nervous systems, the epidermis, and the placodes. In amphibians, these ectodermal tissues develop from the animal pole region of the embryo. During gastrulation, ectoderm covers the whole surface of the embryo. Subsequently, ventral ectoderm develops into the epidermis, whereas dorsal ectoderm gives rise to the neural plate, which is later transformed into the neural tube. The neural tube is further subdivided into the fore-, mid-, and hindbrain and the spinal cord along the anterior–posterior (AP) axis (Nieuwkoop and Faber, 1967). Neural tissue produces neurons and glia, whereas nonneural ectoderm generates ciliated cells, ectodermal glands (e.g., the cement gland, the hatching gland), and the placodes (Nieuwkoop and Faber, 1967).

How is the ectodermal germ layer specified? Are there specific cytoplasmic factors that define ectoderm as opposed to mesoderm and endoderm? Alternatively, ectoderm may develop as a default cell state, when other germ layers are not specified. This review will describe recent studies carried out on Xenopus embryos that reveal the existence of maternal determinants such as Ectodermin, which specifically instruct cells to become ectoderm (Dupont et al., 2005).

The diversification of ectoderm into epidermis and neural tissue is another issue that is addressed in this review. Neural induction, which is also known as *primary embryonic induction,* was first observed by Spemann and Mangold (Harland and Gerhart, 1997). In their experiments, a graft from the dorsal subequatorial region triggered neural plate formation at the ventral side of

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the host embryo. Lineage tracing experiments demonstrated that this ectopic neural plate originated from host ectoderm, thus indicating that it is a result of *induction*. Since the discovery of neural induction, the identification of its mediators has attracted the attention of many embryologists. Recent studies have shown that neural induction involves multiple signaling processes orchestrated by secreted factors of the Wnt, fibroblast growth factor (FGF), and bone morphogenetic protein (BMP) families (De Robertis and Kuroda, 2004; Harland, 2000; Harland and Gerhart, 1997). We review the current understanding of signaling pathways that give rise to neural tissue and epidermis in Xenopus embryos and other vertebrates.

This review will also focus on the mechanisms underlying the differentiation of different cell types: primary neurons from neuroectoderm and ciliated cells from epidermal ectoderm. As in Drosophila, in vertebrate ectoderm, the Notch pathway helps to select individual cells undertaking a specific differentiation pathway. Specific cell types may also form during asymmetric cell divisions as a result of the unequal distribution of cytoplasmic determinants (Bardin et al., 2004). These mechanisms of cell fate specification are distinct from the diversification of epidermal and neural tissue. Finally, we will discuss new research directions that are warranted to further understand ectoderm differentiation in normal development and disease.

I. SPECIFICATION OF ECTODERM AND MESENDODERM BY MUTUALLY ANTAGONISTIC FACTORS

The animal–vegetal axis of the amphibian oocyte forms as a result of the differential deposition of maternal proteins and mRNAs. Embryonic ectoderm is specified in the animal hemisphere, which contains a set of maternally derived factors that differs from the one in the vegetal hemisphere (King et al., 2005). Cell progeny derived from the animal and the vegetal regions of the egg interact via secreted signaling factors to pattern the early embryo and to generate the basic body plan. Molecular mechanisms for ectoderm specification appear to depend on both the inheritance of specific localized cytoplasmic determinants and on cell–cell interactions in the early embryo (Figure 12.1, A through D).

When the ectodermal part of the blastula (animal cap) is excised and cultured in isolation, it develops into atypical epidermis, whereas many secreted polypeptides, known as *mesoderm-inducing factors,* are capable of respecifying this tissue into mesoderm or endoderm (Harland and Gerhart, 1997). These observations suggest that mesoderm and endoderm (mesendoderm) originate from the embryologic default state that corresponds with ectoderm. In agreement with this view, VegT, a T-box transcription factor, was shown to promote mesendoderm formation by activating the transcription of Nodal-related mesoderm-inducing factors of the transforming growth factor beta (TGFβ) superfamily (Zhang et al., 1998). Moreover, antisense oligonucleotide-mediated knockdown of maternal VegT RNA suppresses mesendoderm development and promotes the expansion of ectodermal fates, including epidermis and neural tissue (Zhang et al., 1998). Thus, in the absence of mesoderm- and endoderm-inducing signals, ectoderm appears to develop as a default germ layer. Alternatively, ectoderm may be specified by a localized cytoplasmic factor, and this ectodermal determinant could be missing or suppressed in mesodermal and endodermal tissues.

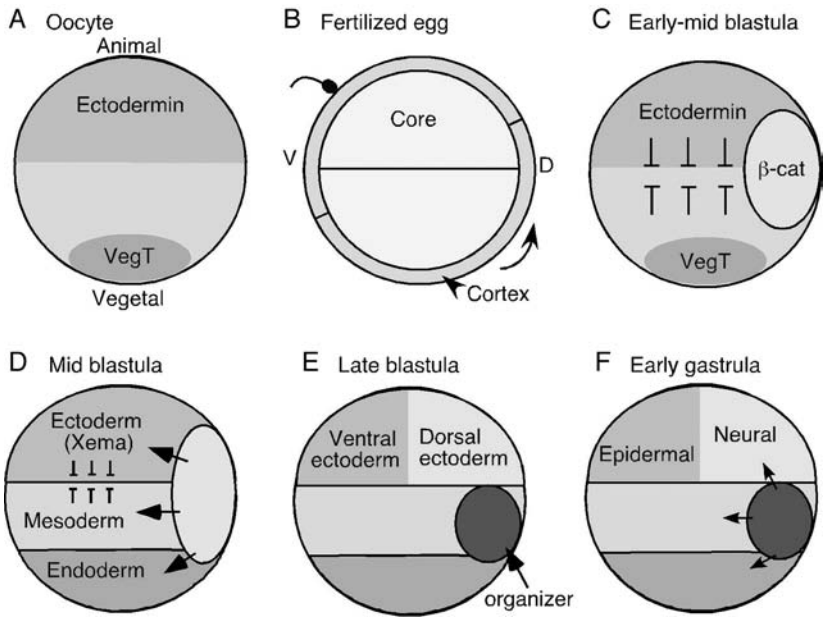

FIGURE 12.1 **Early ectoderm development in Xenopus embryos. A,** During oogenesis, the maternal determinants Ectodermin and VegT are deposited in the animal hemisphere and the vegetal hemisphere, respectively. **B,** After fertilization, the egg cortex rotates relative to the cytoplasm core in a microtubule-dependent process known as the *cortical-cytoplasmic rotation,* which specifies future dorsal and ventral regions of the embryo. The side of sperm entry becomes ventral (V), and the opposite side becomes dorsal (D). **C,** After cortical rotation, β-catenin accumulates on the dorsal side along the animal–vegetal axis. Ectodermin specifies ectoderm by counteracting mesendoderm induced by VegT. **D,** All three germ layers are dorsalized by β-catenin signaling during blastula stages. **E,** Dorsal ectoderm is influenced by β-catenin signaling and becomes predisposed to neural induction. The Spemann organizer forms in the dorsal subequatorial region as a result of the combined action of VegT and β-catenin signaling. **F,** During the early gastrula stage, ventral ectoderm is specified as epidermis by BMP signaling, whereas dorsal ectoderm is specified as neural tissue as a result of BMP antagonists secreted by the organizer.

Recent studies identified gene products that specify ectoderm by suppressing mesendoderm development. Ectodermin, a RING-type ubiquitin ligase, was isolated as a maternal gene product with ectoderm-specific expression (Dupont et al., 2005). Ectodermin promotes the ubiquitination and degradation of Smad4, a protein that is associated with Smad1 and Smad2, which are components of the BMP and Nodal signaling pathways, respectively. Ectodermin inhibits mesendoderm gene markers in presumptive ectodermal tissue and expands *Sox2,* a neuroectodermal marker, presumably by suppressing Nodal and BMP signaling. Supporting this view is the fact that mesendodermal markers are expanded into the animal hemisphere at early gastrula stages in embryos that are depleted of Ectodermin with a specific antisense morpholino oligonucleotide (MO). In these embryos, epidermis is expanded at the expense of neural tissue, and this is consistent with upregulated BMP signaling, which is essential for epidermal development (Dupont et al., 2005; also described later).

Xenopus ectodermally expressed mesendoderm antagonist (Xema) is another recently described protein that is involved in ectoderm specification (Suri et al., 2005). Xema is a Foxi-class transcription factor, which is first

detectable in the ectoderm during the late blastula stages. Xema suppresses mesendodermal gene markers, whereas Xema MOs promote mesendoderm formation in the ectodermal territory. In contrast with Ectodermin, Xema does not influence the conversion of epidermis into neural tissue, thus indicating that ectoderm development is independent from epidermal and neural specification. Because Xema has been proposed to function as a transcriptional activator (Suri et al., 2005), it is expected to induce additional mesendoderm inhibitors. These studies suggest that ectoderm is specified via the inhibition of mesendodermal fates by Ectodermin, Xema, and yet uncharacterized mesendoderm inhibitors. Thus, mutually antagonistic processes operate in early vertebrate embryos to specify ectodermal and mesendodermal cell fates (see Figure 12.1, A through D).

II. SPECIFICATION OF EPIDERMIS AND NEURAL TISSUE

During the late blastula stages, presumptive ectoderm develops into epidermis and neural tissue. This process is closely associated with dorsoventral patterning, because the epidermal tissue is derived from ventral ectoderm, whereas neural tissue forms dorsally (see Figure 12.1). Dorsoventral polarity is generated by the cytoskeletal reorganization (also known as the *cortical-cytoplasmic rotation*), which occurs soon after fertilization (Harland and Gerhart, 1997). Dorsoventral polarization leads to the specification of future neural ectoderm in the dorsal animal region of the embryo and to the formation of the organizer, an early inducing center, in the dorsal vegetal region (see Figure 12.1). The organizer secretes a wide spectrum of specific signaling factors, including BMP antagonists, Wnts, and FGFs, which induce and maintain the developing central nervous system in responding ectoderm (Schohl and Fagotto, 2002; De Robertis and Kuroda, 2004). By contrast, BMP signaling on the ventral side suppresses neural tissue formation and promotes epidermal development (Figure 12.2).

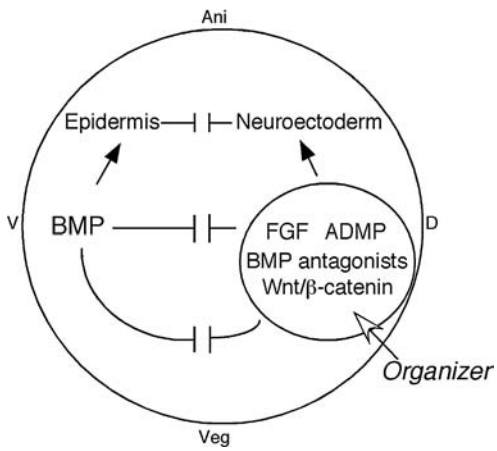

FIGURE 12.2 The regulatory network of pathways involved in neural induction. BMP antagonists function together with Wnt/β-catenin and FGF in the organizer, a dorsal (D) signaling center that specifies neuroectoderm. Ventral BMP signals specify epidermis. ADMP restricts organizer activity. The same pathways antagonize each other throughout the three germ layers. *Ani*, Animal pole; *Veg*, vegetal pole.

A. Responding Tissue

1. Early Specification and Predisposition of Competent Ectoderm to Neural Induction

Early studies have shown that dorsal and ventral ectoderm cells are not equally responsive to organizer-derived signals. When they are isolated before gastrulation, dorsal ectoderm explants are more readily responsive to neural induction than ventral explants (Sharpe et al., 1987). This predisposition may be related to the differential response of dorsal and ventral ectoderm to activin, a TGFβ–like growth factor, observed at blastula stages (Sokol and Melton, 1991). This difference in responsiveness, or *competence*, was attributed to early Wnt/β-catenin signaling, which is responsible for establishing dorsoventral polarity (Sokol and Melton, 1992).

Ectoderm prepattern becomes detectable in dorsal ectoderm before gastrulation as a result of the selective expression of several genes, including *Sox2, Zic1,* and *Xiro1* (Bainter et al., 2001). Other genes, such as *SoxD* and *Geminin,* are expressed throughout ectoderm at blastula stages, and they become restricted to neuroectoderm during gastrulation (Bainter et al., 2001). Both neuroectoderm-specific gene expression and the differential distribution of protein kinase C isoforms (Otte et al., 1991) reflect the labile specification of neuroectoderm, which requires continued organizer signaling for the subsequent development of the vertebrate central nervous system.

The early expression of neuroectoderm-specific genes indicates that neural tissue development is initiated already at blastula stages. Indeed, the Wnt/β-catenin pathway, which is thought to be involved in dorsoventral patterning, is activated soon after fertilization based on the asymmetric nuclear distribution of β-catenin in the dorsal region of the embryo (Harland and Gerhart, 1997). By contrast, active FGF, TGFβ, and BMP signaling pathways assessed with phospho-MAPK and phospho-Smad antibodies are not detectable until mid-blastula stages (Schohl and Fagotto, 2002). A promoter for the early dorsal mesendoderm-specific gene *Siamois* is directly induced by β-catenin, but it fails to be activated by inhibitors of the BMP pathway that are also known to stimulate dorsal development (Fan et al., 1998). These findings suggest that the Wnt/β-catenin pathway functions in dorsoventral specification at an early step, before other signaling pathways (see Figure 12.1).

Whereas the interactions between the major signaling pathways have been studied in some detail with respect to mesoderm development, their role in neural specification is less well known. Because many of these signaling factors influence dorsoventral polarity in all three germ layers (Harland and Gerhart, 1997), the mechanistic relationship between the pathways may be conserved during neural tissue specification. In support of this idea, the dorsal expression of *geminin* is positively controlled by Wnt/β-catenin signaling and negatively controlled by Vent proteins, which are targets of BMP signaling (Taylor et al., 2006). Additional studies of cis-regulatory elements of other early neuroectoderm specific genes are necessary to provide more information about early neural tissue development.

2. Epidermal Development as a Result of Bone Morphogenetic Signaling

The dissociation of blastula ectoderm leads to the suppression of epidermal markers, which can be overcome by exogenous BMP4 (Wilson and Hemmati-Brivanlou, 1997). Similarly, blocking BMP signaling with a dominant negative

BMP receptor inhibited epidermal differentiation and activated neural tissue markers in animal cap explants. Moreover, BMPs 2, 4, and 7 are expressed in the early Xenopus embryo, and they promote epidermal and ventral mesodermal fates (De Robertis and Kuroda, 2004; Harland, 2000; Wilson and Hemmati-Brivanlou, 1997). These findings support a hypothesis that BMP signaling specifies epidermal and antagonizes neural tissue development (Wilson and Hemmati-Brivanlou, 1997).

Mutagenesis screens in zebrafish implicated a large number of BMP signaling mediators in the specification of ventral cell fates, but so far no mutants that completely lose neural tissue have been reported (De Robertis and Kuroda, 2004). Alternatively, a triple knockdown of BMPs 2, 4, and 7 in Xenopus embryos resulted in the significant expansion of neural tissue, but epidermis still developed (Reversade and De Robertis, 2005). This indicates that multiple redundant signaling proteins specify epidermal and neural tissue. In support of this notion, anti-dorsalizing morphogenetic protein (ADMP), a member of the TGFβ superfamily, has been described as possessing BMP-4–like activity (Reversade and De Robertis, 2005). Interestingly, a combined knockdown of ADMP and BMPs 2, 4, and 7 with specific MOs converts the entire ectoderm into neural tissue (Reversade and De Robertis, 2005). Thus, neural induction appears to involve the suppression of BMP signaling and epidermal development (see Figure 12.2).

B. Inducing Tissue: The Organizer

1. Requirement for Organizer in Neural Development

The ability of the organizer (presumptive dorsal mesoderm tissue) to induce ectopic neural plate suggests that it may be essential for neural induction. In support of this notion, the elimination of the organizer in frog embryos by ultraviolet irradiation leads to neural tissue defects (Harland and Gerhart, 1997). Similarly, interference with early Wnt/β-catenin signaling results in embryos that lack the organizer and that are deficient in neural development (Sokol, 1999). Animal pole explants containing presumptive neuroectoderm and lacking dorsal mesoderm fail to develop neural tissue when they are cultured *in vitro* (Harland and Gerhart, 1997). Moreover, in flat dorsal explants containing both the inducing and the responding tissue, neural tissue formation has been shown to entirely depend on the presence of organizer (Holowacz and Sokol, 1999). These studies document the requirement for organizer-derived signals in neural induction in Xenopus.

By contrast, organizer removal by microsurgical extirpation or the *one-eyed pinhead* mutation in the *EGF-CFC* gene, which is required for organizer formation, does not eliminate neural tissue in zebrafish (reviewed by Harland, 2000). Similarly, mechanical removal of the organizer equivalent in chick or mouse embryos did not eliminate neural development. Mouse embryos lacking *HNF-3β,* a gene that is essential for the formation of the node, have neural tissue with essentially correct AP patterning (Harland, 2000). These observations suggest that dorsal mesoderm, which is responsible for neural induction, may partially recover after the operation or that more lateral tissues remaining after the operation retain the capacity to induce neural tissue. Alternatively, organizer may play distinct roles in the neural development of different vertebrate embryos.

2. Neural-Inducing Factors of the Organizer

Since the discovery of the Spemann organizer, the identification of neural-inducing factors has been a major direction of research in vertebrate embryology (Harland and Gerhart, 1997). An important step toward this goal was the identification of molecular markers specific for epidermal and neural cells. For example, Nrp1, Sox2, and NCAM are characteristic of neural tissue, whereas epidermal cytokeratin is a marker for epidermis (Bainter et al., 2001; Harland, 2000). Using these and other markers, several candidate neural inducers were identified. These include Noggin, Chordin, and Follistatin, which are secreted proteins that are capable of inducing neural tissue in animal pole explants (Harland and Gerhart, 1997). These factors antagonize BMP signaling by directly binding BMP ligands. In addition, other secreted BMP antagonists, such as Cerberus and *Xenopus* nodal-related 3, have been reported to trigger neural induction (Harland and Gerhart, 1997). Furthermore, neural-inducing ability has been reported for proteins of Wnt and FGF signaling pathways that are activated in the organizer, which supports the idea that multiple signaling pathways in addition to those of BMP antagonists are involved (see Figure 12.2; De Robertis and Kuroda, 2004; Harland, 2000; Schohl and Fagotto, 2002).

C. Signaling Pathways Regulating Neural Tissue Development

1. BMP Signaling

Studies of the quadruple knockdown of BMP (Reversade and De Robertis, 2005) indicated that BMP signaling should be inhibited for neural induction to occur (see Figure 12.2). During BMP signal transduction, a ligand-activated BMP receptor, which encodes a serine/threonine protein kinase, recruits and phosphorylates Smad1. The phosphorylated Smad1 binds Smad4, and the complex translocates into the nucleus to regulate transcription (Massague and Wotton, 2000). The inhibition of BMP signaling during neural development is accomplished at different levels. Extracellularly, BMP signaling is blocked by the secreted factors Noggin, Chordin, and Follistatin, which directly bind BMPs (De Robertis and Kuroda, 2004). Another level of regulation of the BMP pathway is by the inhibitory Smad6 and Smad7 (Massague and Wotton, 2000; see Chapter 1). The third possible route for BMP signal inhibition is the phosphorylation of the Smad1 linker domain by the MAP kinase (Pera et al., 2003). Finally, BMP gene transcription is known to be suppressed by Wnt and FGF signaling (Delaune et al., 2005; Harland, 2000). Thus, neural tissue development is accomplished via several molecular routes used to inhibit BMP signaling.

Accumulating evidence indicates that neural induction involves multiple cooperating inducing signals. Individual knockdowns of the BMP inhibitors Chordin, Follistatin, and Noggin revealed rather mild defects in neural tissue formation, which suggests that these proteins have redundant functions in Xenopus (Khokha et al., 2005). Consistent with this notion is that the triple knockdown of these BMP inhibitors resulted in dramatic defects in dorsal mesoderm and neuroectoderm, although neural tissue has not been completely eliminated (Khokha et al., 2005). Similarly, mouse embryos with deleted *chordin* and *noggin* genes lack forebrain structures but retain much of the remaining central nervous system (De Robertis and Kuroda, 2004). The incomplete loss of neural tissue in embryos with depleted BMP antagonists indicates that BMP inhibition is only one of several molecular mechanisms involved in neural induction.

Supporting this hypothesis, crosstalk between FGF, Wnt, and BMP pathways has been reported. For example, Smad1 is phosphorylated and regulated by MAP kinase, which is a target of FGF signaling (Pera et al., 2003), whereas BMP-4 transcription is suppressed by β-catenin, which is an essential component of Wnt signaling (Baker et al., 1999). Together, these observations indicate that other pathways in addition to the BMP pathway operate during neural tissue specification (see Figure 12.2).

2. FGF Signaling

The role for FGF signaling in neural induction has been controversial. The FGF pathway includes the tyrosine kinase receptors FGFR-1 through -4, Ras, Raf, and MAP kinase (Eswarakumar et al., 2005). In gain-of-function studies, FGFs have been shown to activate neural tissue markers in partially dissociated ectoderm cells (Harland and Gerhart, 1997), which suggests that FGF enhances the effect of BMP inhibition. Consistent with this view is the fact that neural tissue is readily induced by a combination of BMP antagonists and a low dose of FGF (Delaune et al., 2005). Activated MAPK has been detected in Xenopus neuroectoderm (Schohl and Fagotto, 2002), which suggests that FGF signaling is important for the response of ectoderm to organizer-derived signals. Despite these predictions, blocking FGF signaling with a dominant negative FGFR-1 or Ras constructs failed to affect pan-neural markers (Holowacz and Sokol, 1999; Ribisi et al., 2000). It is possible that these reagents failed to block some FGFR-mediated responses. Indeed, a dominant negative FGFR-4 had a strong suppressive effect on neural development, which suggests that FGFR-4 is specifically involved (Delaune et al., 2005). Additionally, the pharmacologic FGFR inhibitor SU5402 caused a complete loss of neural tissue when added to dorsal ectoderm at blastula stages, thereby indicating an early requirement of FGF signaling before gastrulation (Delaune et al., 2005).

One may consider multiple mechanisms by which FGF signaling affects neural development. First, FGF can interfere with BMP transcription, which was demonstrated by SU5402 upregulating dorsal BMP4 expression (Delaune et al., 2005). Second, FGF may suppress BMP signaling by MAPK-dependent phosphorylation of the linker domain of Smad1 (Pera et al., 2003). However, the role for FGF in neural development is unlikely to be exclusively the result of the inhibition of BMP signaling, because BMP antagonists failed to rescue neural deficiencies caused by SU5402 (Delaune et al., 2005). Thus, accumulating evidence supports the idea that FGF signaling cooperates with BMP antagonists in neural tissue formation (see Figure 12.2).

This involvement of FGF in neural tissue development has been supported by studies in ascidian embryos, in which the role for FGF has been shown to be both necessary and sufficient for neural induction and transcriptional activation of the brain-specific Otx gene homologue (Bertrand et al., 2003). Similarly, FGFs (but not Chordin or Noggin) have been shown to induce neural tissue in the chick embryo (Stern, 2005). Consistent with this is the fact that both dominant interfering FGFR and SU5402 suppressed the node-mediated induction of neural tissue markers (Stern, 2005). Thus, the function of FGF signaling in vertebrate neural induction appears to be conserved.

3. Wnt Signaling

The involvement of Wnt signaling in neural tissue development is not fully clear, partially because this pathway operates in a positive and negative

manner multiple times during embryogenesis. The Wnt pathway involves signaling by Wnt ligands through Frizzled receptors, Dishevelled (Dsh), glycogen synthase kinase 3, β-catenin, and T-cell factors (TCFs) (Sokol, 1999). The overexpression of different pathway components in Xenopus embryos suggests that the activation of Wnt signaling can lead to neural marker stimulation (Baker et al., 1999; Dominguez et al., 1995; Itoh and Sokol, 1997; Sokol et al., 1995). Similar to the FGF pathway, early Wnt/β-catenin signaling inhibits the transcription of BMP4 (Baker et al., 1999) and upregulates the BMP antagonists Chordin, Noggin, and Follistatin (Domingos et al., 2001; Itoh and Sokol, 1997). On the basis of these results, β-catenin has been proposed to predispose dorsal ectoderm to neural induction during blastula stages (see Figures 12.1 and 12.2; Sokol and Melton, 1991; De Robertis and Kuroda, 2004; Stern, 2005). Surprisingly, Wnt signaling has been reported to inhibit neural induction in chick embryos (Stern, 2005), but these observations need to be extended with loss-of-function analysis.

Loss-of-function studies in Xenopus embryos support the essential role for Wnt signaling in neuralization. A dominant negative TCF construct downregulated the pan-neural marker Nrp1 (Baker et al., 1999). In addition, the pan-neural markers Sox2 and Nrp1 are virtually eliminated with MOs against Frodo, a novel scaffold protein involved in Wnt signaling (Figure 12.3; Hikasa and Sokol, 2004). Interestingly, Frodo associates with both Dsh and TCF3, and it is required for TCF-mediated (but not β-catenin–mediated) reporter activation (Hikasa and Sokol, 2004). By contrast, secreted Wnt inhibitors, such as Dkks or FRPs, do not inhibit neural induction (Baker et al., 1999; Itoh and Sokol, 1999; Glinka et al., 1998), which suggests that ligand–receptor interactions are not involved. Curiously, β-catenin MO does not inhibit general neural tissue markers (Heasman et al., 2000; Hikasa and Sokol, 2004). Perhaps β-catenin has to be depleted very early in development for the effect to be observed (Heasman et al., 2000). Although early β-catenin depletion results in ventralization, the effect on axial development is difficult to separate from the effect on neuroectoderm specification. Additional tissue- and stage-specific inactivation studies are warranted to make further conclusions regarding the involvement of Wnt signaling in neural induction.

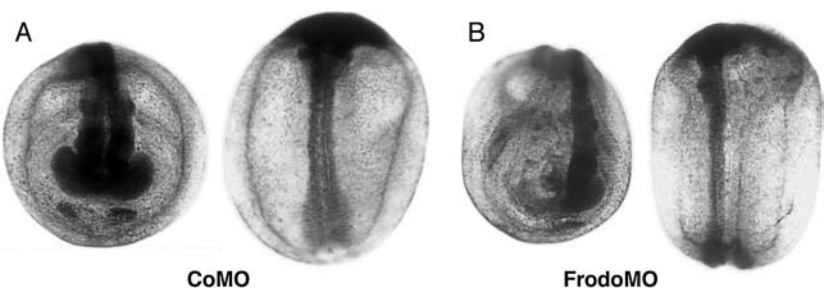

FIGURE 12.3 **A requirement for Frodo in neural development.** Frodo MO *(B)* but not control MO *(CoMO; A)* eliminates neural tissue on one side upon injection into one animal–dorsal blastomere of 8-cell embryos in Xenopus. Whole mount *in situ* hybridization staining for the pan-neural marker *Nrp1* is shown for late–neurula-stage embryos. Anterior (left) and dorsal views (right) are shown for each panel. (See color insert.)

4. Anteroposterior Patterning of Neuroectoderm

The developing central nervous system becomes subdivided along the AP axis into fore-, mid-, and hindbrain and the spinal cord. Nieuwkoop thought that this regionalization was intimately coupled with the process of neuroectoderm specification and proposed the existence of posteriorizing signals acting on newly formed neural tissue (Stern, 2005). This hypothesis has been supported by the demonstration that both FGF and Wnt signals function as posteriorizing signals.

The overexpression of FGF in Xenopus embryo suppresses anterior development and induces posterior neural tissue markers in anterior neuroectoderm (Harland and Gerhart, 1997). In converse experiments, dominant-negative forms of FGFR and Ras and the FGFR-inhibitor SU5402 downregulated posterior neural markers in whole embryos and dorsal explants (Delaune et al., 2005; Holowacz and Sokol, 1999; McGrew et al., 1997; Ribisi et al., 2000). These results indicate that FGFs are involved in patterning neural tissue along the AP axis.

Similar to FGF signaling, Wnt ligands cause anterior head defects (Harland and Gerhart, 1997; Itoh and Sokol, 1999). Anterior neural tissue is transformed into more posterior neural tissue upon the overexpression of Wnt pathway components in Keller explants and neuralized animal caps in Xenopus (Domingos et al., 2001; Harland and Gerhart, 1997; Kiecker and Niehrs, 2001). Reflecting the posteriorizing activity of the Wnt pathway, graded amounts of Dsh specify distinct neural cell fates along the AP axis in animal caps (Itoh and Sokol, 1997). By contrast, negative regulators of Wnt signaling, including β-catenin MO, glycogen synthase kinase 3, and secreted Wnt antagonists, produce anteriorized embryos (Itoh et al., 1995; McGrew et al., 1997; Glinka et al., 1998; Bang et al., 1999; Itoh and Sokol, 1999; Heasman et al., 2000). Together, these studies support a role for Wnts in AP patterning.

Because the Wnt and FGF pathways have been implicated in both AP patterning and neural induction, one needs to consider a possibility that the two processes have a common molecular basis. According to Nieuwkoop, newly induced anterior neural tissue may simply represent the first step in AP patterning. However, the available evidence suggests that neural induction can be experimentally separated from AP patterning. First, the inhibition of FGF signaling by a dominant negative FGFR-1 suppresses posterior neural development but not neural induction (Holowacz and Sokol, 1999), which indicates that posteriorization caused by FGF is different from neuralization. Similarly, secreted Wnt inhibitors inhibit posteriorization, but they do not interfere with neural induction (McGrew et al., 1997). Supporting the view that neural induction is separable from AP patterning is the observation that BMP antagonists induce only anterior neural tissue and that they are unable to stimulate posterior neural markers (Harland and Gerhart, 1997; Wilson and Hemmati-Brivanlou, 1997). The analysis of DNA regulatory sequences in promoters of region-specific and pan-neural genes should prove useful to further understanding the specific molecular mechanisms underlying AP patterning and neural induction.

IV. ECTODERMAL CELL-TYPE SPECIFICATION AND CELL POLARITY

In addition to AP patterning and neural induction, ectoderm undergoes cell differentiation to generate neuronal and glial cells in the central nervous system and also to generate other specialized cells, such as ciliated cells, in the nonneural

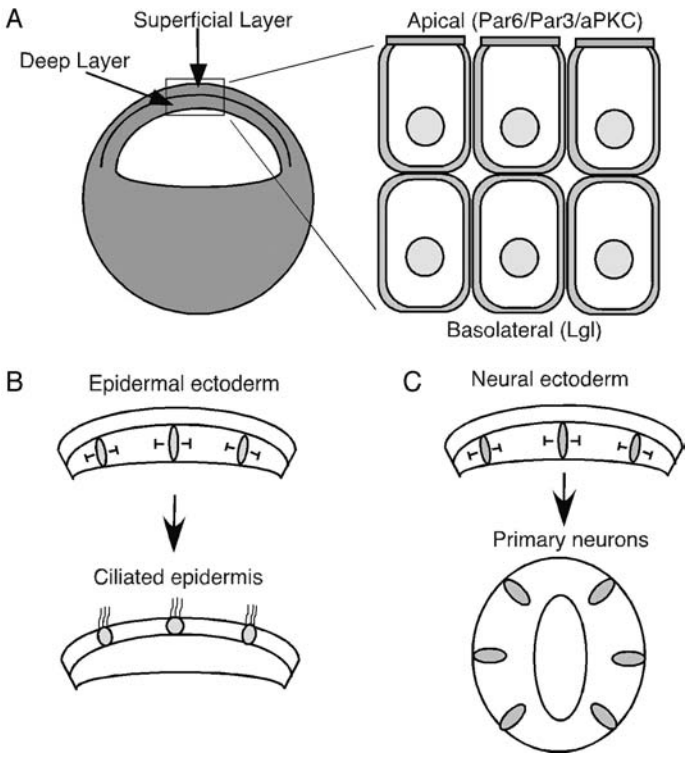

FIGURE 12.4 **The specification of ciliated cells and primary neurons in Xenopus ectoderm. A,** The mid-sagittal section of a late blastula embryo is shown on the left. Animal pole ectoderm is composed of the superficial and the deep layers. The apical complex proteins Par6, Par3, and aPKC (orange) are localized primarily to the apical surface of the superficial layer, whereas the Lgl product (green) is basolateral and present in both cell layers. **B,** Ciliated cells are first specified in the deep layer of epidermal ectoderm in a scattered manner. They then move to the superficial layer during the neurula stages. **C,** Primary neurons are specified in the deep layer of the neural plate to occupy three distinct locations along the dorsoventral axis of the developing neural tube. The specification of both cell types involves inhibitory Notch signaling.

ectoderm of Xenopus embryos (Figure 12.4). The direction of cell differentiation may involve specific signaling pathways, such as the Notch pathway, and it may depend on polarized divisions of epithelial progenitor cells (Bardin et al., 2004). The comparison of neurogenesis in different species reveals a remarkable evolutionary conservation of molecular mechanisms in flies and vertebrates.

A. Different Ectoderm Layers Express Different Molecular Markers and Generate Distinct Cell Types

In *Xenopus* gastrulae, the ectoderm is composed of the superficial and the deep cell layers. The superficial layer has a typical apical–basal polarity, and it is distinct from the deep layer (see Figure 12.4, A). Most primary neurons originate from the deep layer of neuroectoderm (Chalmers et al., 2002; Hartenstein, 1989). During neurulation, the two layers of neuroectoderm intercalate to form a singled-layered neural tube (Davidson and Keller, 1999). In the epidermis, developing ciliated cells migrate from the deep layer to the superficial layer (Deblandre et al., 1999). The molecular mechanisms underlying the differentiation of specific cell types from distinct ectoderm

layers are not fully understood, but they may be hypothesized to derive from the original apical–basal ectoderm polarity (see Figure 12.4).

The apical–basal polarity of epithelial cells is regulated by the *Par* genes, which have been originally identified in the screen for partitioning defective mutations that interfere with the asymmetric division of early *Caenorhabditis elegans* embryos (Bardin et al., 2004). Apical protein complexes, including Par6, Par3, and atypical PKC (aPKC), antagonize the basolateral domain of the epithelial cell, which contains Lethal giant larvae (Lgl) and Par1. The two domains are separated by tight junctions (Bardin et al., 2004). aPKC counteracts Lgl to determine the position of tight junctions in superficial cells and to define the apical–basal polarity in Xenopus ectoderm (see Figure 12.4, A; Chalmers et al., 2005; Dollar et al., 2005). The deep layer of Xenopus ectoderm lacks aPKC, which indicates that it may have originated from asymmetric divisions of animal blastomeres (Chalmers et al., 2005). The Wnt pathway has been implicated in the establishment of the apical–basal polarity, because Dishevelled (a key component of Wnt signaling) functions to regulate Lgl localization (Dollar et al., 2005). Nevertheless, the role for signaling processes in specifying ectodermal layers remains largely unknown. Future studies will address the question of how extracellular signaling controls intrinsic epithelial determinants and specifies cell fate in vertebrate ectoderm.

B. Asymmetric Cell Division and Neuronal Cell Fate Determination in Drosophila

In Drosophila, epithelial polarity proteins—including aPKC/Par3/Par6 and Lgl—are required for the asymmetric distribution of cell fate determinants in neural progenitors, known as *neuroblasts* (Bardin et al., 2004). Neuroblasts delaminate from epithelial cells and retain partial apical–basal polarity. When a neuroblast undergoes asymmetric cell division, a larger daughter cell that inherits apical complex proteins retains the neuroblast fate, whereas the smaller daughter cell differentiates into a neuron or a supporting cell. Thus, in neuroblasts, the apical–basal polarity may be associated with cell fate determination.

The neuroblast fate is also regulated by the Notch signaling pathway, which is conserved in Drosophila and vertebrate embryos. The Notch receptor and the Delta ligand are transmembrane proteins that interact with each other in neighboring cells (Bardin et al., 2004; see Chapter 1). When Notch receives a signal from Delta, the intracellular domain of Notch is cleaved; it translocates to the nucleus, and it associates with Suppressor of Hairless, a DNA-binding protein, to control target gene expression. Only a single progenitor with inactive Notch signaling is selected to differentiate in a group of cells, whereas, in other cells, differentiation is inhibited by the Notch pathway. After neuroblast specification, Notch signaling plays a role in progenitor differentiation by maintaining neuroblasts in an undifferentiated state.

The asymmetric cell division that leads to neuronal fates is causally connected to the Notch pathway. Fly neuronal progenitors, called *ganglion mother cells,* inherit Numb, which is an inhibitor of Notch. Numb is a cell fate determinant that allows neuronal differentiation by inhibiting Notch signaling in one of the daughter cells (Bardin et al., 2004). Thus, the establishment of the apical–basal polarity by aPKC/Par3/Par6 and Lgl, the asymmetric cell division, and the localized distribution of Numb and Notch signaling may represent sequential steps of a conserved pathway that leads to neurogenesis in fly embryos.

C. Neurogenesis in Vertebrate Embryos

Although both the superficial and deep ectoderm layers are competent to neural induction, primary neurons originate from the deep layer only (see Figure 12.4, C; Hartenstein, 1989; Chalmers et al., 2002). ESR-6e, a Notch target that is predominantly expressed in the superficial layer, has been shown to inhibit neurogenesis (Chalmers et al., 2002). This observation suggests that the lack of primary neurons in the superficial layer is the result of active Notch signaling (Chalmers et al., 2002). Additionally, the constitutively active Notch receptor decreases, whereas a dominant-negative form of Delta increases the number of primary neurons within the deep layer (Chitnis et al., 1995; Ma et al., 1996). Thus, the Notch pathway has an inhibitory role in the regulation of neurogenesis in vertebrate ectoderm.

Similar to its role in Drosophila, the apical–basal polarity has retained its conserved function in neurogenesis in vertebrate embryos, presumably by controlling the asymmetric distribution of cell fate determinants, such as Numb. Mice lacking the *Lgl1* gene contain a large number of neural progenitor cells, but they have a decreased number of neurons (Klezovitch et al., 2004). Moreover, a knockdown of AGS3, which is a mammalian protein that is implicated in asymmetric cell division and apical–basal polarity, decreased the number of neural progenitors but stimulated neuronal differentiation (Sanada and Tsai, 2005). Thus, neurogenesis requires the activity of basolateral polarity determinants and the inhibition of Notch signaling; these two molecular systems are not mutually exclusive, but they are likely to function interdependently (Figures 12.4 and 12.5).

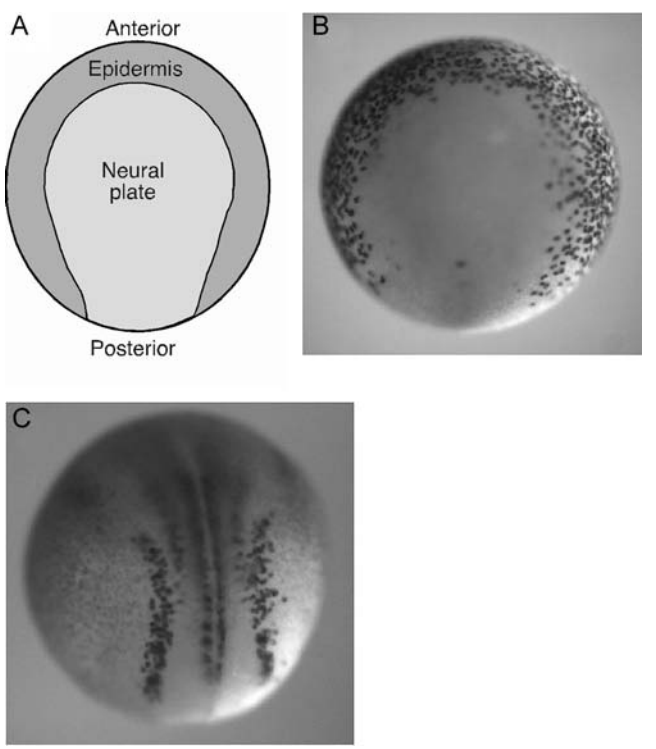

FIGURE 12.5 Specification of ectodermal cell fates during neurulation. Schematic drawing of a *Xenopus laevis* embryo at the neural plate stage. (**A**) Whole mount *in situ* hybridization reveals scattered ciliated cells, characteristic of the deep layer of the epidermis, with an *α-tubulin* probe (**B**) while the three stripes of primary neurons are detected with an *N-tubulin* probe (**C**). (See color insert.)

D. Specification of Ciliated Cells in Epidermal Ectoderm

Xenopus epidermis contains ciliated cells, a specialized cell type that contains a large amount of α-tubulin and that is distributed in an evenly spaced pattern within the superficial layer of nonneural ectoderm (see Figures 12.4, B, and 12.5). Ciliated cells originate from the deep layer of late gastrula and move into the superficial layer during neurula stages (Deblandre et al., 1999). The specification of ciliated cells in the deep layer is controlled by Notch signaling, similar to that seen in the specification of Drosophila neuroblasts and frog primary neurons. Activated Notch decreases and Notch inhibitors increase the number of ciliated cells. The overexpression of ESR-6e, which is a Notch target, also inhibits ciliated cell differentiation, whereas a dominant-negative form of ESR-6e has the opposite effect. Because ESR-6e is mainly expressed in the superficial layer, the specification of ciliated epidermal cells may be negatively controlled by Notch signaling (Deblandre et al., 1999). Thus, the specification of ciliated epidermal cells in the deep layer is tightly associated with the separation of superficial and deep layers of ectoderm as well as Notch signaling (see Figures 12.4 and 12.5).

E. Cell Fate Determination by Separate Patterning Processes

The available evidence indicates that both primary neurons and ciliated cells are specified by cell polarity determinants and by Notch signaling, whereas the specification of epidermal versus neural ectoderm is regulated by BMP antagonists, Wnt, and FGF signaling. Similar to mammalian neuroepithelial cells (Gotz and Huttner, 2005), the polarization of embryonic ectoderm may also play a key role in cell fate specification in Xenopus, with basolateral determinants promoting neuronal differentiation and apical determinants suppressing it (see Figure 12.4). Thus, the molecules and the mechanisms underlying neuronal cell differentiation are conserved between Drosophila and vertebrate embryos (Bardin et al., 2004). We conclude that vertebrate neural tissue develops under the control of separate conserved patterning systems, one involving BMP antagonists and the other based on the unequal segregation of cytoplasmic determinants and Notch signaling between neighboring cells.

The basic knowledge of factors involved in ectoderm development is essential for developing ways to generate specific populations of cells for regenerative medicine. Human neurodegenerative diseases are frequently linked to functional defects in specific cell populations. Basic mechanisms involved in cell polarization and asymmetric cell division may be employed to control the balance over the proliferation and differentiation of progenitor and stem cell populations, and, therefore, they will be important for the design of future stem-cell–based therapies.

SUMMARY

- Germ layers are specified by multiple feedback mechanisms.
- Dorsoventral polarity and inductive signaling influence both the differentiation of ectoderm into neural tissue and epidermis and the AP patterning of the neural tube.

- BMP signaling promotes epidermal development, whereas the inhibition of BMP signaling leads to neural induction. Other patterning factors are expected to function in this process.
- The differentiation of primary neurons and ciliated cells is regulated by asymmetric divisions of polarized ectoderm progenitor cells and by asymmetric Notch signaling.

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We regret that numerous original papers could not be cited as a result of space limitations. We thank Hiroki Hikasa, Maria Krivega, and Olga Ossipova for providing the images used in Figures 12.3 and 12.5 and Jerome Ezan for comments on the manuscript. Work in the Sokol laboratory is supported by the National Institutes of Health.

GLOSSARY OF TERMS

Cytoplasmic determinant
A maternal protein acting to promote specific cell fate.

Epithelial polarity
Regional differences within cells forming an epithelial tissue.

Neural induction
Intercellular communication that converts ectodermal cells into neural tissue.

Neurogenesis
The generation of neurons from uncommitted ectodermal cells.

Organizer
A cell population with an inducing activity.

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13 FORMATION OF THE EMBRYONIC MESODERM

LISA L. CHANG and DANIEL S. KESSLER

Department of Cell and Developmental Biology, University of Pennsylvania School of Medicine, Philadelphia, PA

INTRODUCTION

The developmental mechanisms that control the induction and patterning of the mesodermal germ layer have been studied in a variety of embryonic model systems for decades. These studies have revealed cellular and molecular mechanisms that underlie the induction, patterning, differentiation, and morphogenesis of mesodermal lineages. When defining the cell movements and the inductive signals that control mesodermal development in vertebrates and invertebrates, conserved mechanistic similarities have been identified, as well as interesting and unexpected differences in developmental mechanism. Much has been learned about the formation of the mesodermal germ layer, including the identity of major mesoderm-inducing pathways and the complex regulatory networks that modulate pathway activity. Despite the advances in this research area, many questions are yet to be answered. For example, given that the major mesoderm-inducing pathways regulate distinct lineages throughout development, how is cellular response controlled to ensure an appropriate mesodermal response during early development? Mesoderm formation is a critical embryonic event, and its study has provided profound insight into fundamental developmental mechanisms. Here, we briefly discuss the embryologic and molecular processes that orchestrate the formation of the embryonic mesoderm.

I. INTRODUCTION TO THE EMBRYONIC MESODERM

A. Definition and Embryologic Description

The three primary germ layers—the ectoderm, the mesoderm, and the endoderm—are established during the process of gastrulation. These major lineages are progressively patterned and specialized to give rise to the many

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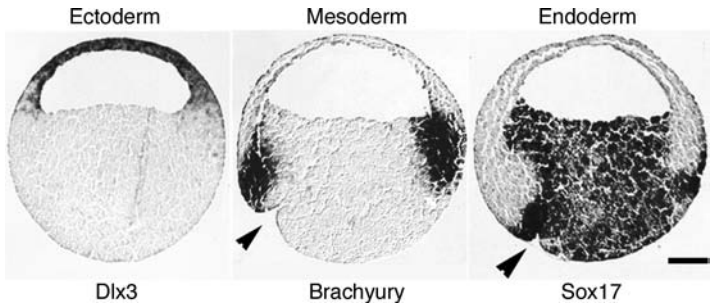

FIGURE 13.1 Formation of the primary germ layers in Xenopus. *In situ* hybridization analysis of sagittal sections of gastrula embryos (animal up and dorsal left). Expression of *Dlx3* in the ectodermal domain (left), *Brachyury* in the mesodermal domain (center), and *Sox17* in the endodermal domain (right). *Arrowhead* indicates dorsal blastopore lip.

tissues and organs that make up the embryonic body plan. The lineages derived from the mesodermal germ layer give rise to a broad range of tissues and organs, including the embryonic tissues prechordal plate, notochord, somites, heart, pronephros, and hematopoietic precursors. In the adult, mesodermal lineages comprise or contribute to skeletal muscle, bone, heart, kidney, blood, dermis, connective tissue, much of the circulatory system, multiple digestive organs, excretory tract, mesenchyme, mesothelium, peritoneum, the reproductive system, and the urinary system (Schier and Talbot, 2005).

In *Xenopus laevis,* the three germ layers form at characteristic positions along the animal–vegetal axis before the onset of gastrulation, with mesodermal precursors positioned as a ring of cells at the marginal or equatorial zone (Figure 13.1). With the onset of gastrulation in Xenopus, mesodermal cells initiate involution movements first at the dorsal marginal zone, which is the position of the Spemann organizer. Mesodermal involution is then initiated progressively in lateral and ventral positions of the marginal zone until the entire mesodermal domain moves internally by the end of gastrulation (Keller, 2002). In zebrafish, the margin of the blastoderm contains mesoderm and endoderm precursors. At the onset of gastrulation, endodermal cells involute first and form the deepest layer of the newly forming hypoblast. Cells near the blastoderm margin that involute early form mesoderm and endoderm, whereas cells that are more distant from the margin involute later and form only mesoderm (Kimmel et al., 1990). Before gastrulation, the mouse embryo is cup shaped, with an internal layer of ectodermal cells (the epiblast) surrounding the proamniotic cavity and an outer layer of visceral endoderm. With the onset of gastrulation, the primitive streak forms at the posterior end of the epiblast and extends toward the distal (future anterior) region of the embryo. Endodermal precursor cells ingress through the primitive streak first, and this is followed by mesodermal precursors, which then occupy the space between the definitive endoderm and the ectoderm (i.e., the epiblast cells that do not ingress; Tam and Behringer, 1997; see Chapter 17).

B. Fate Map and Cell Lineages

Embryonic fate maps identify the position of the precursor cells of differentiated tissues or organs found at later stages of development (Figure 13.2). A fate map describes the potential of a cell or group of cells to adopt a

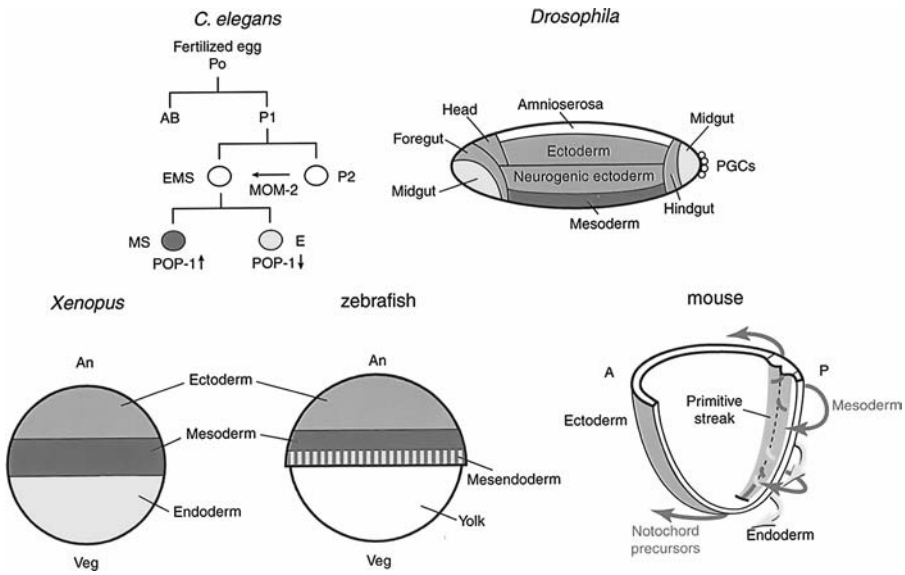

FIGURE 13.2 **Fate maps of the primary germ layers.** Lineage or spatial organization of the primary germ layers in the early *Caenorhabditis elegans* (upper left), Drosophila (upper right), Xenopus (lower left), zebrafish (lower middle), and mouse (lower right) embryos. *Yellow,* endoderm; *red,* mesoderm; *blue,* neuroectoderm. (Figure adapted with permission from Stainier, 2002. See color insert.)

particular fate if left unperturbed in the embryo, but it does not indicate whether a given cell is specified or committed at a particular point in development. Specification occurs when a cell has received signals that instruct its future fate, but that fate choice is not yet permanent or irrevocable. A specified cell adopts its appropriate fate when it is maintained under neutral conditions (e.g., when explanted from the embryo) but not when it is exposed to distinct instructive signals (e.g., when transplanted to an ectopic location in the embryo). By contrast, a determined cell will adopt its appropriate fate, regardless of location of transplantation in the embryo. Although there is much conservation in developmental mechanisms, the coordination of cell division, cell adhesion, cell rearrangements, and the timing of embryonic induction differ among types of embryos, and these differences determine the stage at which meaningful fate maps can be established.

1. Vertebrates

In Xenopus, in which little cell movement occurs before the onset of gastrulation, the initial cleavage divisions define the dorsal–ventral and left–right axes, thus allowing for an accurate assignment of future cell fates in the early cleavage embryo. Detailed Xenopus fate maps have been constructed for the cleavage-stage embryo (16- and 32-cell stages; Dale and Slack, 1987; Moody, 1987a; 1987b). The mesodermal fate map largely reflects the distribution of distinct maternal factors that establish animal–vegetal and dorsal–ventral patterns and that initiate regional gene expression during the mid-blastula stage (see Figures 13.1 and 13.2). The patterning of the mesoderm is further elaborated by zygotic factors produced by cells of the Spemann organizer (dorsal mesoderm) and by competing factors expressed in nondorsal mesoderm (lateral and ventral mesoderm; Heasman, 2006).

The gastrula fate map, with precise boundaries between mesodermal domains (notochord, heart, somites, lateral plate/pronephros, and blood), is a result of the refinement of mesodermal pattern that occurs as a result of zygotic processes.

In zebrafish, extensive cell rearrangements occur during early cleavage divisions, so cell fates cannot be assigned until the onset of gastrulation (Kimmel et al., 1990). Before gastrulation, blastoderm cells form a single homogeneous population. With the onset of gastrulation, clonal groups of the blastoderm cells become fate restricted. Cells initially located near the blastoderm margin involute and give rise to mesodermal and endodermal derivatives, whereas noninvoluting cells farther from the margin form ectoderm (see Figure 13.2; Kimmel et al., 1990; Warga and Kimmel, 1990). After involution, mesoderm is positioned as a ring of cells that includes the dorsal embryonic shield domain. Similar to Xenopus, the mesodermal fate map at the gastrula stage reflects a precise dorsal–ventral organization, with notochord and anterior somite precursors dorsal, pronephros and trunk somites lateral, and tail somites and blood ventral.

In the chick, mesodermal and endodermal precursor cells ingress through the primitive streak during gastrulation, and the mesoderm becomes positioned initially as a loose mesenchyme between the endoderm and the ectoderm. Grafting experiments show that mesodermal cells acquire their identity during gastrulation and that mesodermal commitment occurs after cells exit the primitive streak (Kimura et al., 2006). Mesodermal cells arise along the entire anterior–posterior extent of the primitive streak, with multiple mesodermal cell types arising from a given level of the primitive streak (Psychoyos and Stern, 1996; Schoenwolf et al., 1992).

Fate maps of the mouse at the mid-gastrula stage (embryonic day 7.5) are largely similar to the chick fate map. Rostral levels of the primitive streak, including the node, contain prospective chordamesoderm and paraxial mesoderm (prechordal plate, notochord, and somites); intermediate levels of the streak contain intermediate and lateral plate mesoderm (pronephros and somatic gonad); and more caudal levels contain prospective extraembryonic mesoderm (see Figure 13.2). The striking correspondence of the avian and mammalian mesodermal fate maps reflects the similarities in morphogenetic movements and inductive processes during mesoderm formation (Tam and Behringer, 1997).

2. Invertebrates

Although a comparison of vertebrate and invertebrate fate maps at the gastrula stage reveals some general similarities in the relative positioning of the primary germ layers, dramatic differences in the mechanisms of mesodermal development have been described. In Drosophila, the prospective mesoderm occupies a midventral domain of the blastoderm that is about 18 cell diameters wide (see Figure 13.2). This region invaginates during gastrulation and gives rise to all mesodermal lineages. After internalization, cells of the mesodermal domain rearrange and form a monolayer that then subdivides into different organ primordia, with the anterior mesoderm forming two vertical plates flanking the anterior midgut rudiment and the stomodeum (Stathopoulos and Levine, 2004).

In *Caenorhabditis elegans,* the developmental fate of every somatic cell has been determined. During embryogenesis, 671 somatic cells are generated,

and 113 of these undergo programmed cell death in hermaphrodites. Cell lineage in the embryo and the fate of the resulting differentiated cells are highly invariant (Sulston et al., 1983). Mesodermal lineages are established with the asymmetric division of the EMS blastomere at the 4-cell stage. E and MS, the two daughters of EMS, adopt different fates, with E forming endodermal lineages (intestinal cells) and MS forming mesodermal lineages, including muscles of the pharynx and body wall (see Figure 13.2).

II. MESODERM INDUCTION: EMBRYOLOGY

A. Specification and Determination

Mesodermal development is a progressive process in which the exposure of competent cells to inducing signals during the blastula and early gastrula stages results in the specification of mesodermal fates. Beginning during gastrulation and continuing at later stages, the instructive signals received are interpreted as transcriptional programs that initiate positive and negative regulatory feedbacks, conferring the stability of cell-fate choices and leading ultimately to determination and differentiation. Although embryonic induction involving communication between cell populations via extracellular factors is an inherently cell-nonautonomous process, cell-autonomous factors functioning within mesodermal precursors are required to bias cell-fate decisions during induction and to stabilize mesodermal identity during determination and differentiation. In Xenopus, cells of the marginal zone are competent to form mesoderm in response to mesoderm-inducing signals from the blastula through the mid-gastrula stages. The determination of mesodermal cell fate occurs during the mid to late gastrula stages, and it relies on both cell-autonomous transcriptional processes as well as cell-nonautonomous interactions between mesodermal cells and with the extracellular environment of the embryo (Heasman, 1997; 2006).

The inductive processes that regulate mesodermal development can be categorized as mesoderm-inducing signals and mesodermal-patterning signals, and distinct embryonic signaling centers corresponding to these two functions have been identified in a number of embryos. In Xenopus and zebrafish, mesoderm-inducing signals arise largely from the vegetal pole or yolk cell, whereas the major mesodermal patterning center is the Spemann organizer or embryonic shield. As discussed later in this chapter, the distinction between mesoderm induction and patterning is somewhat arbitrary at the molecular level, with an individual pathway being capable of inducing mesoderm *de novo* but with differing levels of that same signal inducing distinct types of mesoderm. In the embryo, however, the formation and patterning of mesoderm are dependent on the integration of multiple inductive signals that arise in a spatially and temporally dynamic manner with a number of pathways required but with no individual pathway sufficient for normal mesodermal development (Heasman, 1997; 2006).

B. Embryonic Sources of Mesoderm-Inducing Signals

Seminal experiments in the Xenopus embryo (many by Nieuwkoop) defined the source of mesoderm-inducing signals in the blastula and gastrula embryos and identified regions of the embryo that were competent to respond to these signals

and to form differentiated mesoderm. These studies, which are discussed briefly here, identified vegetal pole blastomeres as the source of endogenous mesoderm-inducing signals and the animal hemisphere (including both marginal zone and animal pole cells) as competent to respond to vegetal signals.

1. Vegetal Blastomeres

Consistent with fate-mapping studies, marginal zone explants from the 32-cell stage on autonomously differentiate into mesoderm, whereas explants of the vegetal or animal pole do not (Nakamura and Kishiyama, 1971). In a series of breakthrough experiments, Nieuwkoop found that, although animal and vegetal explants formed ectoderm and endoderm, respectively, when cultured individually, the recombination of animal and vegetal explants resulted in mesoderm formation (Nieuwkoop, 1969a). Further analysis indicated that animal pole tissue was induced to form mesoderm in response to a secreted factor produced by vegetal cells (Dale et al., 1985; Gurdon et al., 1985a; Slack, 1991). These critical experiments identified the vegetal pole as a source of endogenous mesoderm-inducing signals and the animal pole as being competent to form differentiated mesoderm in response to these signals. In subsequent studies, Nieuwkoop showed that, in recombinants containing dorsal or ventral vegetal blastomeres, the dorsal–ventral character of the induced mesoderm reflected the dorsal–ventral source of the inducing vegetal tissue, suggesting that vegetal signals contribute to both mesoderm induction and mesodermal patterning (Nieuwkoop, 1969b). In addition to inducing dorsal mesoderm in animal–vegetal recombinants, dorsal–vegetal blastomeres of the cleavage embryos were shown to induce axial development when transplanted into a host embryo, and came to be referred to as the Nieuwkoop center (Gimlich and Gerhart, 1984). In the zebrafish, the transplantation of the yolk cell and yolk syncytial layer onto the blastoderm results in ectopic mesoderm induction, which suggests a vegetal localization of endogenous mesoderm-inducing signals (Mizuno et al., 1996) that is consistent with the results in Xenopus.

2. Embryo Bisection and Blastomere Removal Studies

Bisection and blastomere removal during early cleavage stages identified regions of the embryo that are sufficient to support axial and mesodermal development. The results of such experiments indicated that the development of axial pattern, including the formation of axial mesoderm, was dependent on the presence of two animal blastomeres and two vegetal blastomeres of the 8-cell stage embryo (Kageura and Yamana, 1983; Kageura and Yamana, 1984). Ligation and blastomere removal studies indicated that mesoderm induction (as assessed by muscle-specific gene expression) was dependent on cytoplasmic material localized to the vegetal hemisphere at as early as the 1-cell stage (Gurdon et al., 1985b). These studies, which were consistent with Nieuwkoop's recombinant studies, indicated that mesoderm-inducing factors are localized to the vegetal pole region of the early cleavage embryo. This conclusion guided later efforts to isolate endogenous mesoderm inducers.

3. Temporal Control of Mesoderm Induction

An essential aspect of embryonic development is the ability to limit the responsiveness of cells both temporally and spatially. This is especially so in

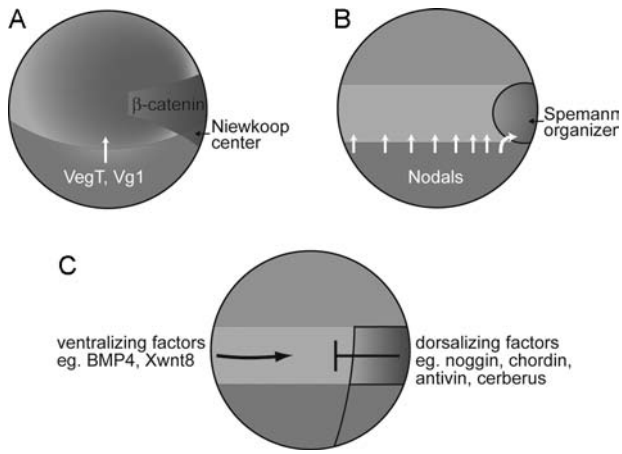

FIGURE 13.3 **Mesoderm induction and patterning in Xenopus. A,** Maternal Wnt signals stabilize β-catenin in dorsal blastomeres, whereas maternal VegT and Vg1 mRNAs are localized to the vegetal pole. The Niewkoop center forms in dorsal–vegetal regions, where dorsalizing signals overlap with vegetal mesoderm-inducing signals. **B,** The initiation of Nodal expression is directly activated by VegT and Wnt signals. Higher levels of Nodal expression in the dorsal marginal zone are essential for the formation of the Spemann organizer. **C,** The interaction of ventral and dorsal signals and their antagonists establish the dorsal–ventral pattern of the mesoderm. The ectoderm is shown at the top of the circles; the mesoderm is shown in the middle; the endoderm is shown at the bottom of the circles. The Spemann organizer and Nieuwkoop center are labeled. (See color insert.)

the case of mesoderm induction, where the cell-nonautonomous character of endogenous mesoderm-inducing factors could result in an expansion of the mesodermal domain if the competence of responsive cells was not limited. As discussed previously, animal pole cells that normally form ectoderm are competent to respond to mesoderm induction, which indicates that spatial limits on competence are not responsible for limiting mesoderm formation to the marginal zone. Alternatively, heterochronic recombinants of animal and vegetal explants indicate that mesoderm induction is regulated by a loss of competence on the part of the animal pole tissue. Although vegetal pole tissue can produce mesoderm-inducing signals from cleavage stages through gastrulation, animal pole tissue loses the ability to respond to inducing signals with the onset of gastrulation (Grainger and Gurdon, 1989; Gurdon et al., 1985a). So, as the mesoderm-inducing signals released by vegetal cells move into the marginal domain, the loss of competence at the beginning of gastrulation establishes the upper (animal) boundary of the mesoderm-forming domain. The loss of competence is an important regulatory mechanism throughout embryogenesis, and an inappropriate maintenance of competence can result in development defects and adult diseases.

III. MESODERM INDUCTION: MOLECULAR MECHANISMS

A. Signaling Pathways

A number of major signaling pathways, including Nodal, Bmp, Wnt, and Fgf, are required for multiple aspects of vertebrate development (Figure 13.3; see Chapter 1). Fgfs and Activin-like members of the transforming growth

factor (TGF)-β superfamily have the ability to induce mesoderm formation in Xenopus animal pole explants. Furthermore, when these signaling pathways are inhibited *in vivo*, mesodermal development is disrupted. Although the stimulation of individual pathways is sufficient to influence mesoderm induction and patterning, cellular integration of multiple signaling inputs and cross-talk between the components of these signaling pathways are essential for the development and differentiation of the germ layers (Candia et al., 1997; Kretzschmar et al., 1997; Nishita et al., 2000). In addition, given the roles of these pathways in many distinct embryonic processes, mechanisms must exist that confer the spatial and temporal specificity of cellular response.

1. Transforming Growth Factor-β Family

More than 40 members in the TGF-β superfamily have been identified in vertebrates and invertebrates. Developmentally important TGF-β subfamilies in vertebrates include TGF-βs, bone morphogenetic proteins (BMPs), Activins, and growth differentiation factors (GDFs). All TGF-β family proteins are synthesized as precursors that undergo cleavage and covalent dimerization of the mature peptide to form secreted biologically active homo- or heterodimers. TGF-β ligands stimulate signaling by binding to heterodimeric receptor complexes with intrinsic serine/threonine kinase activity, and this results in the phosphorylation and activation of Smad proteins that mediate the cellular response (Massague, 1998).

a. Ligands (Activins, Vg1, Nodals, Gdfs, Bmps)

Activin was identified early in the search for mesoderm-inducing factors as a protein that was present in the supernatants of Xenopus tissue culture cells and macrophage cell lines. As a purified protein, Activin is a highly potent inducer of mesoderm, with the ability to induce distinct mesodermal cell types in a dose-dependent manner. At high doses, Activin induces dorsal mesoderm (e.g., notochord); it induces muscle at intermediate doses and ventral–posterior mesoderm at low doses. This response profile suggests that Activin may be a mesodermal morphogen that induces and patterns mesoderm in the early embryo. However, despite the presence of maternal Activin protein, a series of inhibitor studies suggested that Activin was not an endogenous inducer of mesoderm (Kessler, 2004). Recently, the results of Activin knockdown studies provided evidence that Activin is, in fact, required for aspects of mesodermal development. These studies indicate that Activin is required to maintain maximal levels of mesodermal gene expression at the gastrula stage but not for the initial induction of mesoderm (Piepenburg et al., 2004). As a result, Activin knockdown embryos display clear axial defects, but they do not lack axial mesodermal derivatives.

In the mouse, Activin-null embryos do not display early defects in mesodermal development, but embryos null for the receptors that mediate Activin signaling do display severe early defects. These results suggest that early mouse development is dependent on the function of other TGF-β ligands that signal via the Activin receptors but not on Activin function (Kessler, 2004).

Vg1 is encoded by a maternal mRNA that is localized to the vegetal cortex of the Xenopus oocyte. After fertilization, cleavage divisions trap Vg1 protein in vegetal blastomeres, and, therefore, Vg1 has been viewed as

a strong candidate for endogenous mesoderm inducer. However, the native form of Vg1 is not processed efficiently, and, although the mature domain, which is derived from chimeric proteins, can strongly induce mesoderm-like activin, native Vg1 was not found to have significant mesoderm-inducing activity (Kessler, 2004). Recently, a second allele of Vg1 was identified in Xenopus; this form of Vg1 is more efficiently processed and does induce mesoderm. The knockdown of Vg1, like activin knockdown, results in the reduction of mesodermal gene expression during the gastrula stage, but it does not prevent the initial induction of mesoderm (Birsoy et al., 2006). In Xenopus, a second Vg1-related gene, Derriere, is efficiently processed, and it is required for the development of posterior mesoderm but not for the initiation of mesoderm formation (Sun et al., 1999).

Vg1 orthologs and Vg1-like factors have been identified in the zebrafish (DVR1), the chick (cVg1), and the mouse (Gdf1), and, although processed proteins can each strongly induce mesoderm, loss-of-function studies indicate that Vg1 is not essential for the initiation of mesodermal development in these systems. Gdf3, an additional Vg1-like factor in the mouse, is required for early patterning, and Gdf3 nulls display defects in axial patterning and germ layer formation. Given that Vg1-related proteins signal via the same signaling complex as Nodal proteins, it may be that some degree of functional redundancy obscures the early developmental requirement for a subset of Vg1-like proteins (Kessler, 2004).

In contrast with Activin and Vg1, Nodal-related proteins appear to be the critical signaling factors for mesoderm induction in all vertebrates. Loss-of-function, knockdown, and inhibition studies in the mouse, chick, Xenopus, and zebrafish demonstrate the requirement for Nodal function before and during gastrulation for the induction of both the endodermal and mesodermal germ layers (Whitman, 2001). In humans, mice, and chicks, a single Nodal gene is found, whereas zebrafish have three (squint, cyclops, and southpaw); Xenopus has five Nodal-related genes (Xnr-1, -2, -4, -5, and -6) with mesoderm-inducing activity. Most Nodal-related genes are expressed during early embryogenesis in the prospective mesoderm and in the organizer domain.

In Xenopus, Nodal expression is regulated by the maternal factors VegT and β-catenin, and this results in a dynamic expression pattern first in vegetal blastomeres during the late blastula stage, then in the organizer domain during the early gastrula stage, and finally at lower levels throughout the marginal zone during the mid-gastrula stage. Nodal expression in the marginal zone establishes a dorsal–ventral gradient of Nodal signaling activity that contributes to mesodermal patterning. The inhibition of Nodal signals with the specific inhibitor Cerberus-Short results in a complete block of mesoderm induction; this supports the idea that Nodal proteins are the essential initiators of mesoderm formation.

Similarly, Nodal gain- and loss-of-function in the zebrafish result in a dramatic perturbation of mesodermal development (Schier and Talbot, 2005). Embryos that are null for two Nodal genes (cyclops and squint) or the Nodal coreceptor one-eyed-pinhead (mzOep) lack all trunk and head mesoderm as well as endoderm, and they display defects in the initial induction of mesoderm during the gastrula stage (Feldman et al., 2000; Whitman, 2001). As is seen in Xenopus, Nodal gain-of-function induces ectopic dorsal mesoderm and axial duplication in zebrafish (Feldman et al., 1998).

In the mouse, Nodal is expressed in the proximal epiblast before and during early gastrulation, and expression is then restricted to the node. Nodal loss-of-function results in a failure to maintain the primitive streak and a failure to form embryonic mesoderm and extraembryonic ectoderm, and this leads to death during early gastrulation (Conlon et al., 1994; Zhou et al., 1993). Consistent with an essential role in mesoderm formation, loss-of-function for Lefty, a Nodal antagonist, results in an enlarged primitive streak and an excess of mesodermal progenitors. A similar expansion of mesodermal development is observed in Xenopus and zebrafish with the loss of Lefty function.

BMPs function in the early vertebrate embryo to promote ventral mesoderm formation and to limit the domain of dorsal mesoderm formation. BMPs 2, 4, and 7 are expressed in ventral–lateral regions of the early embryo, and they play essential roles in the dorsal–ventral patterning of mesoderm in Xenopus and zebrafish (De Robertis and Kuroda, 2004). BMP gain-of-function suppresses dorsal mesodermal development, whereas the knockdown of BMP function or the overexpression of BMP inhibitors results in an expansion of the dorsal mesodermal domain and the induction of ectopic axial structures (Reversade et al., 2005). Although it is essential for dorsal–ventral patterning of the mesoderm, BMP function is not required for the initiation of mesodermal development. Zebrafish mutants with null alleles of BMP ligands or BMP signaling components are strongly dorsalized, and they show an expansion of axial mesoderm (Hammerschmidt and Mullins, 2002). In the mouse, BMP signaling in the epiblast is essential for the proper recruitment of epiblast cells into the primitive streak, and embryos that are null for the BMP receptor BMPR1A fail to gastrulate or form mesoderm normally.

b. TGF-β Signal Transduction Pathways

On the basis of structural and functional properties, the TGF-β receptors are divided into two families: type I and type II. In the case of Activin-like ligands, these receptors are known as the *Activin-like receptors* (Alks). After the binding of ligand to the extracellular domain of a type II receptor, a type I receptor is recruited into a signaling complex and phosphorylated to activate the serine/threonine kinase activity of the type I receptor. The type I receptor will then phosphorylate and activate Smad proteins, which are the intracellular signaling mediators of TGF-β signaling. Active Smad proteins translocate to the nucleus, complex with specific DNA-binding proteins, and function as coactivators for transcriptional target genes. Three classes of Smad proteins have been identified in vertebrates, including the receptor-activated Smads (R-Smads), Smads 1, 2, 3, 5, and 8, which each contain an SSXS motif that is phosphorylated by active receptor. This R-Smads class is subdivided into two groups based on the types of TGF-β signals transduced, with Activin-like signals mediated by Smads 2 and 3 and BMP-like signals mediated by Smads 1, 5, and 8. A second class includes Smad 4, a collaborating Smad (co-Smad) that forms a complex with each of the activated R-Smads and that is part of the nuclear transcriptional coactivation complex. The third class includes the inhibitory Smads (I-Smads) Smads 6 and 7, which bind to type I receptors and limit the access of R-Smads. Smad 7 negatively regulates a broad range of TGF-β signaling pathways, including Activin-like and BMP-like pathways, whereas Smad 6 specifically regulates only the BMP-like pathways. Additional interactions of Smads 6 and 7 with nuclear factors and other interacting factors

have been reported (Massague et al., 2005). An additional coreceptor protein of the EGF–CFC family (Cripto or one-eyed pinhead) is required for signaling of a subset of TGF-β ligands, including Nodal, Vg1, Gdf1, and Gdf3.

c. TGF-β Signaling Antagonists

Given the potent inducing activity of the many TGF-β ligands expressed in the embryo, it is essential that signaling activity be limited. A number of antagonists of TGF-β signaling have been identified that modulate ligand activity and signaling output. One group of secreted antagonists is structurally related to the Dan protein, including Cerberus and Coco. Xenopus Dan blocks BMP signaling by binding to BMP ligands. Cerberus and Coco are multifunctional inhibitors that block Nodal, BMP, and Wnt signaling by direct ligand binding. A truncated form of Cerberus (Cerberus-Short) is a Nodal-specific antagonist that has been used to demonstrate the requirement for Nodal signaling in Xenopus mesodermal development (Agius et al., 2000; Piccolo et al., 1999). Nodal signaling is also inhibited by Lefty/Antivin-related proteins that prevent the interaction of Nodal ligands with the EGF–CFC coreceptor for Nodal signaling. Tomoregulin-1 can also bind to the Nodal coreceptor to inhibit Nodal signaling. Dapper2 promotes receptor turnover, and BAMBI is a pseudoreceptor that functions in a dominant-negative manner to inhibit signaling by most type I receptors. Smurf1, Smurf2, and Ectodermin are ubiquitin ligases that target Smads 1 and 5, Smad 2, and Smad 4, respectively, for proteosome-mediated degradation. Protein inhibitor of activated STAT (PIASy) associates with Smad proteins in the nucleus to inhibit transcriptional coactivation function.

In addition to Cerberus and Coco, a number of additional inhibitors of BMP signaling are expressed in the organizer domain of vertebrates, including Noggin, Chordin, and Follistatin. Noggin and Chordin bind to BMP-2 and BMP-4 to prevent receptor binding, whereas Follistatin, which also inhibits Activin, can form an inactive complex with BMPs 2, 4, and 7 to inhibit signaling activity. One of the BMP antagonists, Chordin, is subject to negative regulation by Tolloid, a secreted metalloprotease that cleaves Chordin to release bioactive BMP ligands (Connors et al., 1999). In Xenopus, the Tolloid-related protein, Xolloid, cleaves Chordin and Chordin–BMP-4 complexes to limit the inhibition of BMP signaling activity.

2. Fibroblast Growth Factor Family

The fibroblast growth factors (FGFs) comprise a large family of signaling factors that play essential roles in mesoderm induction and maintenance. Purified *Fgf* protein was one of the first proteins identified as a mesoderm inducer in the Xenopus animal explant assay. *Fgf* signaling is crucial as a competence factor in mesoderm induction, and FGF activity is required for the response of animal explants to Activin-like signals. *Fgf*s also regulate the T-box transcription factors that are necessary for the specification and maintenance of mesoderm (see Chapter 16). Dominant-negative, inhibitor, and knockdown studies indicate that *Fgf* signaling is essential during the gastrula stage for the development of trunk and tail structures but not for the initial induction of mesoderm. In addition, studies in zebrafish suggest an early role for *Fgf* signaling in repressing BMP transcription during the late blastula stages to promote dorsal development.

a. Fgf Ligands

In vertebrates, there are at least 23 members of the Fgf family. Fgfs3, 4, 5, and 8 are expressed in mesodermal progenitors of the early mouse. Mutational analysis indicates that, although not all of these Fgf genes are required for mesodermal development, a specific Fgf8 splice variant has been found to have an important role in gastrulation and mesoderm formation (Fletcher et al., 2006). Fgf8 is expressed in epiblast cells and in the primitive streak region and later in the tail bud, which is a primary source of mesoderm at later stages. Fgf4 null embryos die before streak formation, and thus the role of Fgf4 in mesoderm formation is not clear. In Xenopus, Fgf4 is expressed in the blastopore mesoderm during the early gastrula stage, and the stimulation of the Fgf pathway can induce mesoderm formation in explants. The expression of a dominant-negative mutant form of an Fgf receptor (FgfR) results in a loss of trunk and tail structures, including axial mesoderm, but it does not inhibit the initial induction of mesoderm. Similarly, mouse embryos null for FgfR1 display abnormalities in mesodermal patterning during gastrulation and axial defects. These roles for Fgf are likely the result of a positive feedback loop in which Fgf and Brachyury expression are maintained, an interaction required for normal mesodermal development.

b. Fgf Signal Transduction Pathways

Multiple signaling outputs are triggered in response to Fgf binding to its cell surface receptors. The four Fgf receptors (FgfR1–4) have intrinsic tyrosine kinase activity, and, through alternative splicing, numerous FgfR isoforms can be generated. Active signaling is stimulated by the ternary interaction of Fgf ligand, heparan sulfate proteoglycans, and FgfR. Signaling complexes contain a receptor dimer that undergoes autophosphorylation of the cytoplasmic domain, and this results in the recruitment and activation of a series of intracellular effectors of signaling. These downstream effectors include adaptor and docking proteins containing src homology-2 or phosphotyrosine binding domains or signaling enzymes such as protein kinase C (PKC) and the ras/mitogen-activated protein kinase (MAPK) cascade.

c. Fgf Signaling Antagonists

The type I transmembrane protein Sef is a negative feedback inhibitor of Fgf signaling that is coexpressed with and regulated by Fgf ligands. Sef inhibits the activity of FgfR1 and FgfR2 by direct interaction with FgfRs via the Sef intracellular domain. Sprouty2, like Sef, is an Fgf-regulated negative feedback inhibitor of Fgf signaling. Sprouty2 antagonizes calcium-dependent signaling in response to Fgf to inhibit morphogenesis, but it does not inhibit the ras/MAPK pathway or the transcription of mesodermal genes. The related Fgf inhibitor, Sprouty4, interferes with Fgf signaling downstream of the FgfR1 at the level of the ras/MAPK pathway response (Botta et al., 2000).

3. Wnt Family

The Wnt genes constitute a large family of secreted, cysteine-rich, lipid-modified glycoproteins that are involved in many critical processes of early embryonic development (Nusse, 2005). Wnt signaling can stimulate a number of distinct signaling outputs, including the canonical β-catenin–dependent activation of transcription, the planar cell polarity, a calcium-dependent

response, and others. In mesodermal development, the canonical pathway plays several important roles. In Xenopus, cortical rotation during the first cell cycle results in the displacement of dorsal determinants to the future dorsal side of the embryo. The resulting activation of the canonical Wnt pathway stabilizes β-catenin, which accumulates in the nuclei of dorsal blastomeres during the blastula stage and activates the transcription of dorsal gene expression, including Nodal-related genes. Recent evidence suggests that Wnt11 is the maternal ligand that is responsible for the early activation of the Wnt pathway (Tao et al., 2005). During the gastrula stage, zygotic activation of the canonical Wnt pathway results in the stabilization of β-catenin in ventrolateral regions, which promotes the developmental of ventral–posterior mesoderm and antagonizes dorsal–anterior mesoderm. Therefore, the canonical Wnt pathway is first used during the maternal phase of development to promote dorsal fates and then to promote ventrolateral fates in response to zygotic signals.

a. Wnt Ligands

Although Wnt ligands were at one point categorized into canonical and noncanonical signaling classes, it now appears that an individual Wnt ligand can stimulate distinct signaling output, depending on the repertoire of receptors, cofactors, and signaling components present in responding cells. In Xenopus and zebrafish, Wnt8 is the major zygotic ligand responsible for ventrolateral patterning of the mesoderm. Zygotic gain of function for Wnt8 antagonizes organizer function and results in dorsoanterior defects in axial development. Zygotic Wnt8 signals via β-catenin to activate the ventrolateral expression of Vox and Vent transcriptional factors that promote ventral gene expression and repress dorsal genes. The inhibition of zygotic Wnt8 function causes an expansion of dorsoanterior structures, including enlarged head structures and the reduction of tail and trunk.

Maternal depletion studies show that Wnt11 is essential for dorsal development in Xenopus. Maternal Wnt11 signals via β-catenin to activate dorsal gene expression, including the expression of organizer-specific genes, and this suggests that Wnt11 is the endogenous signal that is responsible for the initiation of dorsal development. Consistent with this, Wnt11 mRNA is enriched in the dorsal blastomeres of the early cleavage embryo, presumably as a result of cortical rotation (Tao et al., 2005).

In Xenopus and zebrafish, Wnt signals do not induce mesoderm formation, but they are essential for the dorsal–ventral patterning of the mesoderm and axial development. In the mouse, Wnt3 is expressed in the proximal epiblast before gastrulation, and a Wnt3–null mutant lacks a primitive streak and fails to form mesoderm (Liu et al., 1999). Therefore, in contrast with Wnt function in Xenopus and zebrafish, early Wnt signals are essential for mesoderm formation in the mouse.

b. Wnt Signal Transduction Pathways

Frizzled proteins are a family of seven-pass transmembrane serpentine receptors that transduce Wnt signals. Wnt ligands bind to the extracellular cysteine-rich domain of Frizzled proteins that signal in conjunction with a low density lipoporotein receptor-related protein (LRP) coreceptor. The ternary complex of Wnt–Frizzled–LRP results in the phosphorylation of

Dishevelled, a cytoplasmic mediator of signaling, which subsequently inhibits the multifunctional serine/threonine kinase, GSK3β. The inhibition of GSK3β permits the accumulation of β-catenin, which translocates to the nucleus and interacts with T-cell factor/lymphoid enhancer factor (TCF/LEF) family transcription factors to activate the transcription of downstream target genes. In the absence of ligand binding, cytoplasmic β-catenin interacts with APC and Axin scaffold proteins and is a substrate for phosphorylation by CKI and GSK3β. Phosphorylated β-catenin is then ubiquitinated and destroyed by the proteosome. In the absence of nuclear β-catenin, TCF/LEF factors recruit Groucho corepressors to repress the transcription of Wnt target genes (Nusse, 2005). Although other outputs of the Wnt pathway play essential roles in the morphogenesis of the mesoderm, the canonical pathway described here is the output responsible for Wnt regulation of mesodermal patterning.

c. Wnt Signaling Antagonists

Antagonists of Wnt signaling include Frzb, Dkk1, Cerberus, and Crescent. In addition, secreted Frizzled-related proteins (sFRP) contain a cysteine-rich domain, but lack the transmembrane domain and therefore bind Wnt ligands in the extracellular space and prevent signaling. Dickkopf-1 (Dkk1) is a secreted protein expressed in the organizer that inhibits zygotic Wnt signaling to promote the formation of head structures. Dkk1 binds to LRP coreceptors and promotes their endocytosis and depletion from the plasma membrane, thus inhibiting the cellular response to Wnt signals. Frzb and Cerberus are secreted factors that are expressed in the organizer and the anterior mesendoderm, respectively, and that directly bind Wnt ligands to inhibit signaling. Like Dkk1, Frb and Cerberus promote dorsoanterior development and inhibit trunk and tail development, which is consistent with the ability to block zygotic Wnt signaling.

B. Mesodermal Transcriptional Networks

1. T-box Family (Brachyury, VegT, and Eomesodermin)

T-box transcription factors mediate the response to multiple developmental signaling pathways, including the TGF-β and FGF pathways (Naiche et al., 2005). T-box proteins play essential roles in the establishment and patterning of the primary germ layers in the vertebrate embryo as well as in later aspects of limb development and organogenesis (see Chapter 16). Among a number of T-box proteins that are specifically involved in early mesodermal development are Brachyury, VegT, and Eomesodermin.

The founding member of the T-box family is the mouse T/Brachyury gene (Herrmann et al., 1990). In the mouse gastrula, Brachyury is expressed in the primitive streak and node, in mesodermal precursor cells, and in the notochord. Brachyury is required during gastrulation for the mesodermal specification of cells of the epiblast, just before their ingression through the primitive streak. In the absence of Brachyury function, prospective mesoderm cells fail to ingress through the primitive, and gastrulation is disrupted. Brachyury-null mice die shortly after gastrulation, and they have several mesodermal abnormalities, including a shortened and thickened primitive streak and the absence of posterior mesoderm (Beddington et al., 1992).

Brachyury orthologs in Xenopus (Xbra) and zebrafish (no tail) are similar in expression pattern and developmental function. In Xenopus, Xbra expression is initiated in the marginal zone of the late blastula; it is maintained in the prospective mesoderm and in the closing blastopore throughout gastrulation, and it is expressed in the axial mesoderm forming the notochord (Smith et al., 1991). Xbra gain of function is sufficient for the induction of ventrolateral mesoderm in animal explants, and, in cooperation with dorsalizing factors such as Noggin, Xbra can induce dorsal mesoderm (Cunliffe and Smith, 1992; Cunliffe and Smith, 1994). The inhibition of Xbra function results in a number of mesodermal defects, including a failure to express mesodermal genes, a defect in the convergent extension movements of gastrulation, and the death of posterior mesodermal cells, which are similar to the defects observed in the Brachyury null mice (Conlon et al., 1996; Conlon and Smith, 1999). Xbra expression in the marginal zone is initiated in response to Nodal signals, and an Xbra–Fgf positive feedback loop maintains both Xbra expression and Fgf signaling in the mesoderm of the marginal zone and blastopore (Isaacs et al., 1994).

In the zebrafish gastrula, no tail is expressed in the mesodermal precursors of the germ ring before and during gastrulation and in the notochord. Zebrafish no-tail mutants, like Brachyury mutants in the mouse, have early defects in mesodermal development and severe posterior axial defects, and fail to form a notochord (Halpern et al., 1993; Schulte-Merker et al., 1994). No-tail expression is regulated by Nodal and Fgf signals, and it interacts with these pathways to regulate posterior development (Griffin and Kimelman, 2003; Schier et al., 1997). Mesodermal development in the zebrafish is dependent on a regulatory network of partially redundant T-box genes (including no tail, spadetail, and tbx6) that function together to regulate the formation of axial and paraxial mesoderm and the subsequent formation of trunk and tail structures (Amacher et al., 2002; Goering et al., 2003; Griffin and Kimelman, 2003).

VegT is a maternally encoded T-box transcription factor that is localized to the vegetal pole of the Xenopus oocyte (Zhang and King, 1996). Zygotic VegT is expressed by mesodermal precursors in the marginal zone and in the posterior paraxial mesoderm. The depletion of maternal VegT mRNA results in a failure to form the endodermal and mesodermal germ layers, an expansion of ectoderm, and a loss of the zygotic expression of multiple Nodal-related genes (Kofron et al., 1999; Zhang et al., 1998). VegT gain of function results in an expansion of mesoderm and endoderm and in the ectopic expression of Nodal. The transcription of multiple Nodal genes is directly activated by VegT. Given its essential role in the development of the germ layers and the onset of Nodal expression, VegT is often referred to as a master regulator of endodermal and mesodermal development.

In *Xenopus*, Eomesodermin (Eomes) is zygotically expressed before other pan-mesodermal genes in a dorsal–ventral gradient in the prospective mesoderm, with the highest levels in the organizer domain. Eomes gain of function activates mesodermal genes, whereas the inhibition of Eomes function results in gastrulation arrest and defects in mesodermal gene expression (Ryan et al., 1996). Eomes is maternally expressed in zebrafish, and it is localized in a vegetal-to-animal gradient; zygotic Eomes protein is localized to the nuclei of dorsal blastomeres. The overexpression of zebrafish Eomes results in the formation of ectopic dorsal mesoderm and secondary axial structures, and the

response to Eomes is dependent on the Nodal signaling pathway (Bruce et al., 2003). Eomes is required for the formation of endoderm and mesoderm in the zebrafish, and, as a maternal factor, it is an essential regulator of germ-layer formation, similar to VegT in *Xenopus* (Bjornson et al., 2005). In the mouse, Eomes is expressed in the oocyte, the preimplantation embryo, and the blastocyst, and loss of function results in gastrulation defects with a failure to recruit mesodermal cells to the primitive streak (Russ et al., 2000). Taken together, these observations indicate that Eomes is an essential regulator of the earliest stages of mesodermal development and that this function is conserved in vertebrates.

2. Fox Family (Fast1, FoxD3)

The Fox gene family comprises a large and functionally diverse group of forkhead-related transcriptional regulators that are essential for metazoan embryogenesis, and multiple Fox genes have been implicated in mesodermal development (Lehmann et al., 2003). Forkhead Activin signal transducer (Fast1 or FoxH1) is an essential mediator of the transcriptional response to Activin-like signals (Stemple, 2000). Fast1 forms a complex with Smads 2 and 4 in response to Activin-like signals such as Nodal, and this complex binds to target gene promoters to activate transcription (Attisano et al., 2001; Chen et al., 1996; Chen et al., 1997). In addition, the Fast1–Smad2–Smad4 complex mediates Nodal autoregulation via conserved intronic binding sites (Osada et al., 2000). Experiments in Xenopus, zebrafish, and mouse have established Fast1 as a key regulator of Nodal signaling. The inhibition of Fast1 function in Xenopus results in the failure to form the organizer and a partial loss of mesodermal gene expression (Watanabe and Whitman, 1999). The zebrafish Fast1 mutant, schmalspur, displays mesodermal and axial defects similar to the cyclops/squint double mutant, with a loss of notochord and a reduction of dorsal mesodermal gene expression (Pogoda et al., 2000; Sirotkin et al., 2000). Similarly, Fast1-null mice fail to form the node, the prechordal mesoderm, and the notochord, but they do form some mesoderm (Hoodless et al., 2001).

Although these loss-of-function studies strongly support an essential role for Fast1 in the transcriptional response to Nodal signals, the formation of a small amount of mesoderm in Fast1 mutants suggests that residual Nodal signaling may occur via a Fast1-independent pathway. Alternatively, the mechanisms of Nodal target gene regulation by Fast1 may be more complex than signal-dependent activation. Current models of Fast1 function suggest that, in the absence of Smad-2–activating signals, Fast1 is a quiescent factor that lacks transcriptional activity. A more complex view of Fast1 function comes from a recent Fast1 knockdown study in Xenopus (Kofron et al., 2004). The maternal depletion of Fast1 resulted in the predicted loss of expression for most Nodal target genes. However, two Nodal-related genes, Xnr5 and Xnr6, were expressed at elevated levels in Fast1 knockdown embryos. This surprising result suggests that Fast1 may negatively regulate a subset of target genes, thus indicating a more dynamic role for Fast1 in Nodal signaling and mesodermal development.

FoxD3 has multiple roles in the vertebrate embryo, including the regulation of neural crest development and the maintenance of mammalian stem cell lineages. FoxD3 is also expressed in the Spemann organizer of Xenopus, the

zebrafish shield, and the node of the chick and mouse (Labosky and Kaestner, 1998; Odenthal and Nusslein-Volhard, 1998; Yaklichkin et al., 2003; Yamagata and Noda, 1998). In recent work, it has been demonstrated that FoxD3 function in the Spemann organizer is essential for dorsal mesodermal development. FoxD3 functions as a transcriptional repressor to induce dorsal mesoderm and axis formation, and the antagonism or knockdown of FoxD3 results in severe axial defects and a loss of dorsal mesodermal gene expression. FoxD3 induction of mesoderm is cell nonautonomous, and it requires the Nodal signaling pathway. Consistent with the coexpression of FoxD3 and Nodal genes in the organizer, FoxD3 is necessary and sufficient for the expression of several Nodal-related genes. Taken together, these results indicate that FoxD3 maintains Nodal in the Spemann organizer by repressing a negative regulator of Nodal expression, thus promoting mesoderm induction and axis formation (Steiner et al., 2006; Yaklichkin et al., 2007). Similarly, zebrafish FoxD3 functions in the shield to promote Nodal expression, dorsal mesoderm formation, and axial development (Chang and Kessler, unpublished).

3. Transcriptional Antagonists

Multiple Nodal antagonists have been identified that act at each step of the Nodal signal transduction cascade. At the transcriptional level, antagonists limit the activity of transcriptional coactivators, the transcription of Nodal genes, or the transcription of Nodal target genes. Xenopus PIASy is a SUMO ligase that is expressed in the animal hemisphere and in a dorsal–ventral gradient in the marginal zone, with the highest levels ventral (Daniels et al., 2004; Sachdev et al., 2001). PIASy directly interacts with Smad 2 to inhibit transcriptional coactivation function. The knockdown of PIASy results in an expansion of the mesodermal domain into the animal pole region and the formation of ectopic dorsal mesoderm in the ventral marginal zone (Daniels et al., 2004). Similarly, the ubiquitin ligase Ectodermin is localized to the animal pole, and it destabilizes Smad 4 to inhibit Nodal signaling. Ectodermin gain of function suppresses mesoderm formation, whereas knockdown causes an expansion of the mesoderm domain into the animal pole (Dupont et al., 2005). These Smad antagonists limit mesoderm formation to the marginal zone by suppressing Nodal signaling in the animal pole, and they contribute to the dorsal–ventral patterning of the mesoderm by moderating Nodal pathway activity in the marginal zone.

The nuclear factors Sox3, Zic2, and Drap1 inhibit the expression of Nodal genes to limit mesodermal development. In Xenopus, the maternal factors Sox3 and Zic2 inhibit the expression of Nodal genes. Maternal Sox3 is distributed in an animal-to-vegetal gradient with the highest levels at the animal pole. Interference with Sox3 function in Xenopus and zebrafish results in gastrulation defects, the expansion of mesoderm, and the upregulation of Nodal expression. In Xenopus, Sox3 functions as a repressor to directly regulate the transcription of Xnr5 (Zhang et al., 2004). Maternal Zic2 is uniformly distributed in the gastrula. Similar to Sox3, Zic2 knockdown results in defects in gastrulation and axis formation and in the upregulation of Nodal genes and mesodermal genes in the gastrula (Houston and Wylie, 2005). Although the results indicate that Zic2 suppresses Nodal expression to limit mesoderm formation, the mechanism of Zic2 function (including transcriptional activity and targets) is yet to be determined. In the mouse, Drap1 is expressed throughout the epiblast during

gastrulation; and Drap1 null embryos display severe gastrulation defects, the expansion of Nodal expression, and the upregulation of Nodal target genes. Drap1 binds to the forkhead domain of Fast1 to prevent DNA binding, thus preventing Nodal positive feedback (Iratni et al., 2002). These antagonists limit the intensity and spatial extent of Nodal transcription in the gastrula embryo to restrict mesodermal development and to maintain the proper organization of the germ layers.

Xenopus ectodermally expressed mesendoderm antagonist (Xema) is a FoxI-related gene that is zygotically expressed in the animal domain of Xenopus. Xema overexpression inhibits mesoderm formation in the marginal zone and suppresses axis formation consistent with a loss of dorsal mesoderm. In animal pole explants, Xema inhibits mesoderm induction by both Activin and Fgf, which suggests that it does not specifically regulate an individual pathway but rather that it may act to suppress the response of downstream mesodermal genes. Conversely, Xema knockdown results in ectopic mesoderm formation and axial dorsalization in the embryo and the *de novo* formation of mesoderm in animal explants. Interestingly, Xema functions as a transcriptional activator to suppress mesodermal development; this suggests that Xema may act indirectly by activating specific target genes to inhibit the expression of mesodermal genes (Suri et al., 2005). Taken together, the transcriptional antagonists discussed in this section provide a regulatory system for limiting the activity of and response to mesoderm-inducing pathways, thus maintaining the spatial organization of the germ layers that is essential for normal development.

IV. THE SPEMANN ORGANIZER AND MESODERMAL PATTERNING

After the initiation of mesoderm induction at the blastula stage, patterning of mesodermal lineages begins with a process of inductive signaling between mesodermal cells within the marginal zone. The primary signaling center responsible for dorsal–ventral patterning of the mesoderm during gastrulation is the Spemann organizer. In this section, a brief overview of the process of organizer formation and of the developmental and molecular functions of the organizer in mesodermal patterning is provided.

A. Cortical Rotation and the Nieuwkoop Center

In Xenopus, fertilization breaks the radial symmetry of the egg and results in a reorganization of the cortical and deep cytoplasm in a process of cortical rotation (Harland and Gerhart, 1997; Moon and Kimelman, 1998). This displacement of the cortical cytoplasm relative to the deeper cytoplasm during the first cell cycle establishes the dorsal–ventral asymmetry of the early embryo, with dorsal opposite the site of sperm entry. During cortical rotation, dorsal determinants initially located at the vegetal pole are transported to the equatorial region to confer dorsal identity. The disruption of cortical rotation blocks the establishment of dorsal–ventral polarity, and the resulting "ventralized" embryos fail to form dorsal mesoderm or axial structures. Such ventralized embryos can be rescued during the first cell cycle by tipping the embryo, which displaces the dorsal determinants from the vegetal pole to establish dorsal–ventral polarity (Gerhart et al., 1989).

The earliest evidence of dorsal–ventral patterning is observed in the inductive properties of the vegetal pole. Nieuwkoop found that the type of mesoderm induced in animal–vegetal recombinants reflected the dorsal–ventral origin of the vegetal tissue (Boterenbrood and Nieuwkoop, 1973; Nieuwkoop, 1969b). Gerhart and colleagues extended these observations in a series of blastomere transplant studies in the early cleavage embryo. Dorsal–vegetal blastomeres of the 32- and 64-cell–stage embryos could induce axis formation when transplanted to the ventral–vegetal region of a host embryo, and this resulted in the formation of an ectopic axis in a normal host or in the rescue of axis formation in a ventralized host. Lineage mapping indicated that the transplanted blastomeres did not contribute to the induced axial tissues but rather to the anterior endoderm of the gut (Gimlich and Gerhart, 1984). Therefore, the dorsal–vegetal cells were capable of inducing adjacent marginal zone cells to form the Spemann organizer. These results indicated that cortical rotation confers a dorsal inductive property to dorsal–vegetal blastomeres and this early signaling center of the cleavage embryo was designated as the Nieuwkoop center (see Figure 13.3). An analogous process appears to regulate dorsal development in the zebrafish. Asymmetrical cytoplasmic streaming carries dorsal determinants from the yolk cell to the yolk syncytial layer of the future dorsal side of the embryo, and signals from this extraembryonic tissue induce adjacent blastomeres of the dorsal margin to form the shield/organizer (Chen and Kimelman, 2000; Jesuthasan and Stahle, 1997; Schneider et al., 1996).

A series of studies have shown that cortical rotation activates a maternal Wnt pathway that is essential for dorsal determination (Moon and Kimelman, 1998). The overexpression of Wnt ligands in the ventral–vegetal blastomeres of a normal embryo or in the vegetal blastomeres of a ventralized embryo can induce the formation of the Spemann organizer and axial development. In addition, the knockdown or inhibition of multiple Wnt pathway components, including the intracellular effector β-catenin, can ventralize the embryo in the same way as blocking cortical rotation can (Wylie et al., 1996). The link between cortical rotation and the activation of Wnt signaling was confirmed by the observation that maternally supplied components of the Wnt signaling pathway (including Wnt 11, Dishevelled, and β-catenin) become asymmetrically localized to dorsal blastomeres in a cortical-rotation–dependent manner (Larabell et al., 1997; Miller et al., 1999; Schneider et al., 1996; Tao et al., 2005). A number of direct transcriptional targets of β-catenin are activated in dorsal blastomeres at or just before the mid-blastula transition, including Siamois, Twin, Xnr3, Xnr5, and Xnr6, and these genes play important roles in the formation of the Spemann organizer. Similarly, in the zebrafish, β-catenin directly activates Squint and Bozozok in the dorsal yolk syncytial layer and in the dorsal marginal blastomeres.

The Nieuwkoop center is defined experimentally by the ability of dorsal–vegetal blastomeres to induce organizer formation when transplanted to a ventral–vegetal position of a host embryo. However, no molecular asymmetry—either in localized mRNA or protein—has been identified that uniquely marks the Nieuwkoop center blastomeres. Instead, dorsal determinants are distributed in a broader animal–vegetal region that includes the dorsal marginal zone that forms the Spemann organizer (Kikkawa et al., 1996). So, although transplanted dorsal–vegetal cells can induce organizer formation without contributing to organizer tissue, in the unmanipulated embryo,

endogenous organizer-inducing signals are not limited to dorsal–vegetal cells, but are also found in the precursor cells of the Spemann organizer. Therefore, organizer formation may not be a sequential process in which dorsal determinants of the Nieuwkoop center induce a spatially distinct group of cells to form the organizer; rather it may be a more direct process in which dorsal determinants are present in the cells of the cleavage embryo that will form the organizer. Consistent with this idea is the observation that the formation of the zebrafish shield occurs even after the elimination of the yolk syncytial layer (Chen and Kimelman, 2000).

B. Formation and Function of the Spemann Organizer

1. Organizer Transplantation and Organizer Genes

A fundamental experiment in the history of embryology was the organizer transplant of Spemann and Mangold (Spemann, 1938; Spemann and Mangold, 1924). In this dramatic demonstration of embryonic induction, the transplantation of a gastrula dorsal blastopore lip (the mesoderm of the dorsal marginal zone) to the ventral marginal zone of a host embryo resulted in the formation of an ectopic body axis with complete anterior–posterior and dorsal–ventral organization. In early experiments, pigmentation differences between embryos allowed for discrimination between host cells and transplanted donor cells, and the examination of the tissues formed indicated that much of the ectopic axis was formed from host tissue. Thus, the dorsal blastopore lip could "organize" host tissues, which normally form ventral mesoderm and epidermis, into a complete body axis through a process of cell–cell communication or induction. The organizer transplant experiment was reexamined using modern lineage-mapping techniques, and this confirmed that, although the donor tissue formed the chordamesoderm (the notochord and the prechordal plate) of the induced axis, paraxial mesoderm, neural, and endodermal structures were derived from host tissue (Smith and Slack, 1983). In the fish, chick, and mouse, transplants of the shield or node (the anatomical equivalent of the dorsal blastopore lip) also resulted in the induction of an organized body axis (Beddington, 1994; Oppenheimer, 1936; Storey et al., 1992). Embryologic studies in multiple systems have defined three conserved aspects of organizer function: (1) cells of the organizer undergo convergent extension movements to drive axial elongation; (2) organizer tissue differentiates to form chordamesoderm (prechordal plate and notochord); and (3) the organizer regulates the developmental patterning of adjacent tissues by inductive signaling (see Figure 13.3) (De Robertis and Kuroda, 2004; De Robertis et al., 2000, 2001; Harland and Gerhart, 1997).

The identification of the organizer as a conserved signaling center in the vertebrate gastrula stimulated intense efforts to define the molecular mechanisms of organizer function. The factors and pathways that mediate organizer formation and function have been identified by expression screens for organizer-specific genes, by functional screens for genes that mimic organizer function, and by the testing of candidate genes and pathways for organizer activity. The initial breakthrough in defining the molecular components of the organizer came from De Robertis and colleagues, who screened an organizer cDNA library and identified Goosecoid, a homeodomain transcription factor expressed specifically in the Spemann organizer (Blumberg et al., 1991; Cho et al., 1991). Such differential screening strategies have identified

a number of important organizer genes, including Chordin (Sasai et al., 1994), and genomic approaches have recently been used to identify additional organizer genes (Hufton et al., 2006; Wessely et al., 2004). Harland and colleagues established a functional screening strategy in which the sib-selection of cDNA pools was used to identify genes that induce organizer formation or that mimic organizer function. Among the important organizer genes identified in functional screens are Noggin, Xnr3, and Siamois (Lemaire et al., 1995; Smith et al., 1993; Smith et al., 1995). In addition, candidate approaches have identified multiple components of the Wnt and Nodal signaling pathways as inducers of organizer formation (Jones et al., 1995; McMahon and Moon, 1989; Sokol et al., 1991). Overall, several dozen organizer-specific genes have been identified, and their study in multiple model systems provides striking evidence of the conservation of the organizer at the molecular level. For example, in all vertebrate embryos examined, Goosecoid is expressed in the organizer domain during the early gastrula stage, thus providing a powerful tool for visualizing organizer formation. However, there are a few examples of species-specific organizer genes; this suggests a degree of evolutionary variation in the mechanisms of organizer formation, which is discussed later in this chapter.

2. Maternal Wnt Signals

The combined action of maternal and zygotic signals is required for the formation of the Spemann organizer at the onset of gastrulation (see Figure 13.3). In gain-of-function studies, organizer formation can be induced by either dorsalizing signals or mesoderm-inducing signals. The activation of the maternal Wnt pathway by gain of function for Wnt ligands, Frizzled, LRP, Dishevelled, or β-catenin or by the inhibition or knockdown of Axin, GSK3β, or APC can induce organizer formation (Moon and Kimelman, 1998; Nusse, 2005). A number of transcriptional targets of the maternal Wnt pathway have been identified that are implicated in organizer formation or function. The transcription of these target genes, including Siamois, Twin, Xnr3, Xnr5, and Xnr6, in dorsal blastomeres at the mid-blastula transition is a direct transcriptional response to β-catenin–Tcf3 (Brannon et al., 1997; Fan et al., 1998; Hilton et al., 2003; Laurent et al., 1997; McKendry et al., 1997; Rex et al., 2002; Takahashi et al., 2000). Although Xnr5 and Xnr6, like other Nodal genes, induce dorsal mesoderm, Xnr3 has distinct functions in convergent extension and neural induction (Hansen et al., 1997; Takahashi et al., 2000; Yokota et al., 2003). Siamois and Twin are closely related paired-type homeodomain proteins that are identical in their regulation by maternal Wnt signals and their ability to mimic organizer activity (Brannon and Kimelman, 1996; Carnac et al., 1996; Fan et al., 1998; Laurent et al., 1997; Lemaire et al., 1995). Inhibition and knockdown studies indicate that Siamois and Twin are functionally redundant proteins that together are essential for organizer formation and for organizer induction by the Wnt pathway (Fan and Sokol, 1997; Kessler, 1997; Bae and Kessler, unpublished). Siamois and Twin activate the transcription of organizer genes during the early gastrula stage, and they are essential for mediating the organizer-specific transcriptional response to maternal Wnt signals. For several organizer genes, including Goosecoid and Lim1, it has been shown that Siamois and Twin directly activate transcription by binding to homeodomain sites within Wnt-responsive

promoter elements (Fan and Sokol, 1997; Kessler, 1997; Yamamoto et al., 2003). Therefore, maternal Wnt signals promote organizer formation in Xenopus by activating "organizer formation" genes via β-catenin during the mid-blastula stage and "organizer function" genes via Siamois and Twin during the early gastrula stage.

Interestingly, although Siamois and Twin are essential for the transcriptional control of organizer formation in Xenopus, orthologs have not been identified in other vertebrate model systems. This suggests that Siamois and Twin represent a recent regulatory innovation in Xenopus (and other amphibia) acting downstream of β-catenin in organizer formation. Despite differences in developmental mechanisms suggested by the absence of Siamois and Twin orthologs, the Siamois–Twin binding site of the Xenopus Goosecoid promoter is conserved in mouse, chick, and zebrafish Goosecoid (Bae and Kessler, unpublished), which suggests that a Siamois–Twin-like factor may play an equivalent role in organizer formation in other systems. In the zebrafish as well, a regulatory innovation has occurred downstream of β-catenin in the form of Bozozok, a homeobox gene found only in the zebrafish. Bozozok is directly activated by β-catenin in the dorsal yolk syncytial layer, where its function is essential for organizer formation (Fekany et al., 1999; Koos and Ho, 1999; Solnica-Krezel and Driever, 2001; Yamanaka et al., 1998). In contrast with Siamois and Twin, Bozozok promotes organizer formation as a transcriptional repressor that prevents the expression of ventral genes, such as BMP2b and Wnt8, in the dorsal organizer domain (Fekany-Lee et al., 2000; Koos and Ho, 1999; Leung et al., 2003). The isolation of Siamois, Twin, and Bozozok orthologs, if any, or the identification of functionally equivalent transcriptional regulators of organizer formation will require further efforts.

3. Zygotic Nodal Signals

Organizer formation is also induced by Smad-2–activating TGF-β ligands, including Activin, Nodal, Derriere, Vg1, Gdf1, and Gdf3. At doses sufficient to strongly activate Smad 2 and to induce dorsal mesoderm, each of these factors can induce axis formation (Chen et al., 2006; Jones et al., 1995; Sun et al., 1999; Thomsen et al., 1990; Thomsen and Melton, 1993; Wall et al., 2000). Similarly, gain of function for downstream signaling components, including Smad 2 and a constitutively active form of Alk4, induces organizer formation and secondary axial structures (Chang et al., 1997; Graff et al., 1996; Hoodless et al., 1999). Consistent with the ability of Smad 2 pathway activation to induce organizer formation, the inhibition of endogenous signaling components (ActRIIB, Alk4, Smad2, and Fast1) by dominant negatives, knockdown, or natural inhibitors blocks organizer formation and axial development (Chang et al., 1997; Hemmati-Brivanlou and Melton, 1992; Hoodless et al., 1999; Watanabe and Whitman, 1999). These studies indicate that Smad-2–dependent TGF-β signaling is essential for organizer formation. However, not all of the TGF-β ligands capable of inducing organizer formation are required for the formation of the endogenous organizer. In Xenopus, the knockdown or inhibition of Activin, Derriere, or Vg1 does not inhibit organizer function (Birsoy et al., 2006; Dyson and Gurdon, 1997; Hemmati-Brivanlou et al., 1994; Piepenburg et al., 2004; Sun et al., 1999). So, although Activin, Derriere, and Vg1 can induce organizer formation and each plays an

important role in mesodermal development, these are not the endogenous inducers of organizer formation.

Nodal function is essential for organizer formation in all vertebrates (Schier, 2003; Whitman, 2001). Inhibitor studies in Xenopus and loss-of-function analyses in the zebrafish and mouse have demonstrated the requirement for Nodal function in organizer formation. In Xenopus, a dominant-negative Xnr2 mutant or the Nodal-specific inhibitor Cerberus-Short inhibits organizer formation, thereby resulting in severe gastrulation and axial defects (Agius et al., 2000; Osada and Wright, 1999; Piccolo et al., 1999). Zebrafish doubly mutant for cyclops and squint fail to form the shield, and dorsal cells do not undergo involution during gastrulation (Feldman et al., 1998), and the mouse Nodal mutant fails to form the node or gastrulate (Conlon et al., 1994; Zhou et al., 1993). Although these results indicate that organizer formation is dependent on Nodal function, the organizer defects could be interpreted as an indirect consequence of the failure to induce mesoderm. However, a direct role in organizer formation is indicated by the observation that Nodal genes in Xenopus, zebrafish, and mouse are expressed in the organizer domain coincident with the formation of the organizer at the onset of gastrulation (Whitman, 2001). In Xenopus, the mesodermal expression of Xnr1, Xnr2, and Xnr4 is initiated in the dorsal marginal zone just before the start of gastrulation, and the activation of Smad 2 in the organizer domain confirms the presence of active Nodal signaling (Agius et al., 2000; Lee et al., 2001). For several organizer genes, including Goosecoid and Lim1, it has been shown that the transcriptional response to Nodal signals is directly mediated by DNA-binding factors, either Fast1 (FoxH1) or Mixer, which form a transcriptional activation complex with Smads 2 and 4 that binds to defined promoter elements (Germain et al., 2000; Kunwar et al., 2003; Watabe et al., 1995; Watanabe et al., 2002; Yamamoto et al., 2003). Therefore, Nodal signals induce mesoderm during the late blastula stage, and elevated Nodal expression in the dorsal marginal zone at the onset of gastrulation is essential for organizer formation.

The study of the inductive pathways that regulate organizer formation indicates that both maternal Wnt signals and zygotic Nodals signals are required for organizer formation (see Figure 13.3). Mechanistic analyses of the interaction between the Wnt and Nodal pathways indicate that the signaling outputs of these pathways are integrated at the transcriptional level, thereby resulting in the cooperative activation of organizer genes. Thus, the combined action of these pathways on dorsal blastomeres establishes a transcriptional domain in which organizer genes are expressed. The mechanism of signal integration at the level of organizer gene transcription appears to require, at least in some cases, the close proximity of Wnt- and Nodal-responsive elements in organizer gene promoters (Watabe et al., 1995; Yamamoto et al., 2003). For example, in the Xenopus Goosecoid promoter, Nodal- and Wnt-response elements are closely spaced (within 100 bp), and mediate a synergistic transcriptional response to the combination of Nodal and Wnt signals (Watabe et al., 1995; Bae and Kessler, unpublished). Although the precise mechanism of this transcriptional synergy is not yet defined, it seems likely that cooperative binding to the response elements or the cooperative recruitment of common transcriptional coactivators may mediate this interaction between the Nodal and Wnt pathways. Strikingly, closely spaced Nodal- and Wnt-response elements are present in the promoters of mouse and zebrafish

Goosecoid, which suggests that signal integration via coupled response elements may represent a conserved mechanism for establishing the organizer transcriptional domain (McKendry et al., 1998; Watabe et al., 1995). A direct interaction between components of the Nodal and Wnt pathways (Smad4–β-catenin–Tcf3) has been identified in the regulation of Siamois–Twin transcription, and this suggests a transcriptional cooperation in this context as well (Nishita et al., 2000). Furthermore, the activation of a subset of organizer genes by Siamois–Twin is dependent on cooperation with Nodal-like signals (Darras et al., 1997; Engleka and Kessler, 2001). Therefore, multiple cooperative interactions between the Nodal and Wnt pathways are required for the regional activation of organizer gene transcription.

3. The Organizer as A Source of Signaling Antagonists

The ability of the transplanted organizer to influence the development of adjacent tissues indicated that the organizer functions in a cell-nonautonomous manner, via inductive signals, to pattern the mesoderm and organize the body axis. A fundamentally important and unexpected outcome of the molecular analysis of the organizer was the discovery that the organizer is a source of secreted antagonists of multiple signaling pathways rather than a source of positive-acting instructional signals. So, rather than producing signals that directly confer dorsal identity of adjacent tissue, a key function of the organizer is to produce inhibitors of pathways that promote ventral identity, thus permitting the dorsal development of adjacent tissues. The function of these signaling antagonists in developmental patterning of the mesoderm is to exclude pathway activity from the organizer domain and to produce an activity gradient in the nonorganizer mesoderm, with the highest levels at the ventral marginal zone (De Robertis, 2006; De Robertis and Kuroda, 2004; Harland and Gerhart, 1997).

The primary targets of inhibition by the organizer are the BMP and zygotic Wnt pathways, which are active in the gastrula and which promote the formation of ventral–posterior mesoderm (see Figure 13.3). Organizer-specific signaling antagonists have been identified in differential expression screens, functional screens, and by candidate approaches, as discussed previously. In general, these factors were found to mimic organizer activity and to promote dorsal fates and axial development. In all vertebrates, BMP signals play an essential role in the development of the ventrolateral mesoderm and the nonneural ectoderm. Experiments in Xenopus, zebrafish, and mouse have identified multiple BMP antagonists expressed in the organizer. These antagonists (Noggin, Chordin, Follistatin, Cerberus, and Xnr3) inhibit the activity of the BMP signaling pathway and promote dorsal development (Fainsod et al., 1997; Hansen et al., 1997; Iemura et al., 1998; Piccolo et al., 1999; Piccolo et al., 1996; Zimmerman et al., 1996). In Xenopus, the overexpression of any one of these BMP inhibitors can convert ventral mesoderm into dorsal cell types and induce ectopic axial structures. In the case of Noggin, Chordin, Follistatin, and Cerberus, biochemical studies have shown that direct binding to BMP ligand prevents receptor activation and signaling. The mechanism of BMP inhibition by Xnr3 is less clear, but it may involve an interaction of the Xnr3 prodomain with BMP proteins to inhibit processing and maturation. BMP antagonists secreted by cells of the organizer spread into adjacent tissues and establish a dorsal–ventral gradient of BMP signaling

activity that patterns the mesoderm (Dosch et al., 1997). It should also be noted that the neural induction activity of the organizer is largely a result of the antagonism of BMP signaling in the prospective neural plate (De Robertis and Kuroda, 2004). The importance of BMP antagonism in organizer function is confirmed by the observation that the simultaneous knockdown of Noggin, Chordin, and Follistatin results in a dramatic loss of dorsal mesoderm, an expansion of ventral and posterior mesoderm, and the failure to form a neural plate (Khokha et al., 2005).

The canonical Wnt pathway has distinct maternal and zygotic roles in mesodermal and axial development. As discussed above, maternal Wnt signals induce organizer formation and the development of a complete body axis. By contrast, Wnt signaling after the mid-blastula transition promotes posterior axial development and inhibits head formation (Kiecker and Niehrs, 2001). A number of Wnt antagonists are produced by the organizer, including Frzb1, sFRP2, Crescent, Cerberus, and Dickkopf1, and these secreted proteins promote dorsal–anterior development and the formation of head structures (Niehrs, 2004). Frzb1, sFRP, and Crescent are structurally related to the extracellular ligand-binding domain of Frizzled, and they prevent signaling by direct binding to Wnt ligands (Leyns et al., 1997; Pera and De Robertis, 2000). Cerberus also binds directly to Wnt ligands to inhibit signaling (Piccolo et al., 1999). Dickkopf1 antagonizes Wnt signaling by binding to LRP6, a Wnt coreceptor, and promoting the clearance of Wnt receptor complexes from the cell surface (Glinka et al., 1998; Mao et al., 2001). As with BMP antagonists, secreted Wnt inhibitors diffuse into adjacent tissues and generate a Wnt activity gradient that confers anterior–posterior pattern. In addition to the signaling antagonists produced by the organizer, transcriptional repressors, including Goosecoid in Xenopus and Bozozok in zebrafish, are expressed within the organizer; and these proteins directly repress the transcription of BMP and Wnt genes (Fekany-Lee et al., 2000; Ferreiro et al., 1998; Koos and Ho, 1999; Leung et al., 2003; Yao and Kessler, 2001). Therefore, the inhibition of ventral pathways by the organizer involves cell-nonautonomous mechanisms via secreted signaling antagonists and cell-autonomous mechanisms by direct transcriptional repression.

Interestingly, inhibitors of the Nodal pathway are also secreted by the organizer, which is itself a source of Nodal signals. These antagonists include Cerberus, which binds Nodal ligands, and Lefty and Antivin, which are related proteins that bind to the Nodal coreceptor Cripto/one-eyed pinhead to inhibit signaling (Cheng et al., 2000; Juan and Hamada, 2001; Piccolo et al., 1999). These factors are feedback inhibitors of signaling that limit the intensity and spatial extent of Nodal pathway activity. Taken together, these observations reveal that the organizer is the source of a multifunctional cocktail of signaling inhibitors that modulate the activity of the BMP, Wnt, and Nodal pathways. In fact, there is a strong correlation between the combined activities of the BMP, Wnt, and Nodal pathways and the anterior–posterior organization of the body axis. Head development is dependent on the inhibition of all three pathways; trunk development requires the inhibition of BMP signaling with active Wnt and Nodal signaling; and tail development requires active signaling by all three pathways (Glinka et al., 1997; Niehrs, 2004; Piccolo et al., 1999). Therefore, the signaling antagonists produced by the organizer establish a dynamic signaling environment within the gastrula that controls the spatial organization of the vertebrate body plan.

V. METAZOAN MESODERM FORMATION: THEME AND VARIATION

A. Vertebrates

In this chapter, a discussion of mesoderm induction and patterning has been presented; it has focused primarily on vertebrates, with particular attention to studies in Xenopus that have revealed detailed mechanisms of mesodermal development. Although there are differences between model systems in the mechanisms of mesodermal development, the conclusion from work in this area it that there is much in common mechanistically, and these similarities reveal fundamental aspects of mesodermal development. These commonalities include an essential role for maternal and zygotic pathways, including inductive signaling by Nodal, BMP, Wnt, and FGF. In addition, the organizer, which forms in response to Nodal and Wnt signals, is a source for multiple signaling antagonists that pattern the mesoderm to establish the proper organization of the body axis. When examining the mechanisms of mesodermal development in invertebrate model systems, some similarities to vertebrate mechanisms are found, but there are also significant differences. Here a brief overview of mesodermal development in several invertebrate systems is provided.

B. Invertebrates

1. Caenorhabditis elegans

In *C. elegans*, mesodermal lineages are largely derived from two distinct cell lineages (MS and ABa) that form during early cleavage stages. The MS lineage forms mesodermal precursors that differentiate into the muscle of the body wall and posterior pharynx. MS is generated as a result of an asymmetric division of EMS at the 4-cell stage. The daughters of EMS (E and MS) differ with regard to the maintenance of Pop-1 levels, a Tcf family transcription factor that promotes mesodermal specification. EMS receives a Wnt signal from P2, an adjacent posterior cell, and, as a result Pop-1 is downregulated in E, which then adopts endodermal fate. By contrast, MS, the anterior daughter, does not receive a Wnt signal; it maintains Pop-1 levels and forms mesoderm (Thorpe et al., 1997). At the 12-cell stage, descendants of ABa come into contact with MS and receive a Notch-activating signal. The transcriptional response to Notch signaling in the ABa lineage is dependent on two redundant T-box genes, Tbx37 and Tbx38, that specify the mesodermal fate of this lineage, which forms the muscle of the anterior pharynx (Good et al., 2004). TGF-β signals do not appear to play an essential role in the development of embryonic mesoderm in *C. elegans*. Therefore, mesodermal development in *C. elegans* is regulated in two distinct ways, with the Tcf-related factor Pop-1 promoting mesodermal fate in the MS lineage and the Notch activation of T-box genes promoting mesodermal development in the descendants of ABa.

2. Drosophila

Mesodermal development in Drosophila is dependent on inductive signals and cell-autonomous regulators that specify distinct mesodermal cell types. The maternal factor, Dorsal, establishes the dorsal–ventral pattern of the early embryo, defines the boundaries of the mesodermal domain, and promotes Dpp expression in the dorsal ectoderm. Just before the invagination of the prospective mesoderm, Tinman, an Nkx-class homeodomain transcription

factor, is activated in prospective mesodermal cells in response to Twist. During germband extension, invaginated mesoderm comes into contact with the dorsal ectoderm, thereby exposing mesodermal precursors to Dpp, a BMP-related factor, which cooperates with Tinman to specify mesodermal cell fates. Wingless, which is a Wnt family protein, interacts with the Dpp–Tinman pathway to confer anterior–posterior pattern on the mesoderm. Wingless cooperates with Dpp to induce dorsal vessel (cardiac) and somatic muscle, and it antagonizes Dpp signals to induce visceral mesoderm, thereby establishing the proper dorsal–ventral and anterior–posterior organization of mesodermal structures (Campos-Ortega and Hartenstein, 1997; Frasch and Nguyen, 1999).

3. Sea Urchin

In the sea urchin, mesoderm is derived from two cell populations of the cleavage embryo, the primary mesenchyme cells (PMCs) and the secondary mesenchyme cells (SMCs). The PMCs are derived from micromeres and ingress into the blastocele, whereas the SMCs are derived from macromeres and give rise to several distinct mesodermal cell types. The induction of both mesodermal lineages, as well as endodermal lineages, is dependent on the vegetal expression of Wnt 8, which induces the accumulation of nuclear β-catenin in a vegetal domain that includes both micromeres and macromeres. Interestingly, the requirement for nuclear β-catenin in mesodermal lineages is transient, and, after hatching, β-catenin is downregulated in both PMCs and SMCs to permit mesodermal differentiation (Croce and McClay, 2006). Similar to the formation of mesoderm derived from the ABa lineage of *C. elegans,* Notch signaling is essential in the sea urchin for the formation of the SMCs. Nuclear β-catenin activates the expression of the Notch ligand, Delta, in micromeres, and this stimulates a Notch signaling response in the macromeres, thus promoting SMC formation (Sherwood and McClay, 1999). A Nodal ortholog is expressed in the sea urchin, but, unlike Nodal expression in vertebrates, Nodal is expressed in the ectoderm of the sea urchin before gastrulation, and it regulates the formation of the oral–aboral axis (Duboc et al., 2004). So, although similar signaling pathways (Wnt–β-catenin and Nodal) regulate mesoderm and axis formation in both sea urchins and vertebrates, the specific developmental roles of these pathways differ dramatically.

4. Ciona

The ascidian Ciona is a member of the urochordate clade, which is thought to represent the closest living form to the ancestral chordate (Satoh, 2003). The larval swimming tadpole form contains several mesodermal derivatives, including the notochord, muscle, and mesenchyme. The notochord lineage is induced at the 32-cell stage by adjacent endodermal cells, which is similar to mesoderm induction in Xenopus. A Brachyury ortholog expressed in the notochord lineage is both necessary and sufficient for notochord formation. Like the sea urchin, nuclear β-catenin accumulates in endodermal cells and activates a number of genes, including several FGF genes that are essential for the induction of Brachyury and notochord formation (Kim et al., 2000; Satoh, 2003). Nodal signals in Ciona do not play a role in the induction of the primary notochord at the 32-cell stage, but they are required for the induction of the secondary notochord, which forms the notochord of the

posterior body axis. Nodal signaling from lateral mesenchyme induces Brachyury expression and the differentiation of the secondary notochord lineage (Hudson and Yasuo, 2006). It is striking that this "ancestral" form makes use of developmental mechanisms that are similar to those described in sea urchin (β-catenin–positive endodermal signaling center) as well as in vertebrates (Nodal regulation of notochord development).

VI. CONCLUSIONS AND PERSPECTIVES

The formation of the vertebrate body plan is a process of self-organization, with the fertilized egg undergoing subdivision and induction to set up the primary germ layers and organizing centers, leading to morphogenesis, differentiation, and axis formation. Localized maternal determinants establish the spatial organization of the blastula and initiate regional gene expression. Although maternal factors bias cell fate, zygotic transcriptional programs are required to determine cell fate and confer stable embryonic pattern. During gastrulation, these transcriptional networks undergo positive and negative feedback, which reinforces lineage-specific gene expression and refines boundaries between developmental compartments. In this way, developmental programs are selected and maintained in the gastrula, thereby providing a stable spatial framework for further elaboration of the body plan.

The study of mesodermal development has provided a rich experimental paradigm for defining molecular mechanisms of development. The great success of research in this area has provided a depth of understanding of the embryologic and molecular mechanisms of mesoderm induction and patterning. In particular, a wealth of knowledge has been obtained regarding the embryonic signaling pathways that control mesodermal development. These studies have not only provided fundamental insight into developmental mechanisms, but they have also defined novel mechanisms of signal transduction. What is less well understood at this point is the mesodermal transcriptome: the complete expression profile of genes expressed in the developing mesoderm and the genome-wide identification of the transcriptional targets of the embryonic signaling pathways that induce and pattern mesoderm. With the completion or near completion of genome sequences for all of the major model systems, it will now be possible to obtain complete transcriptome data sets by microarray-based expression profiling of wild-type, mutant, and knockdown embryos. By coupling these data with chromatin immunoprecipitation approaches and promoter arrays, a detailed description of the transcriptional networks underlying mesodermal development is now within reach. Efforts are currently underway to define the transcriptome and transcriptional networks of the mesoderm (Kimelman, 2006), and the coming years should yield fundamental insights into the embryonic development of the mesoderm.

SUMMARY AND FUTURE DIRECTIONS

- Localized maternal factors establish polarity in the egg and activate the zygotic expression of embryonic inducers of mesoderm.

- Major signaling pathways, including the TGF-β, FGF, and Wnt pathways, play important roles in mesoderm induction and patterning.
- Nodal ligands are essential for the induction of mesoderm in vertebrates.
- Nodal and Wnt signals cooperate to induce the formation of the organizer in the gastrula.
- The organizer is a source of secreted antagonists of BMP and Wnt signaling that regulate the dorsal–ventral and anterior–posterior patterning of the mesoderm.
- Transcription factors of the Fox, T-box, and homeodomain families are activated by mesoderm induction, and these proteins constitute a transcriptional network that controls mesodermal patterning and differentiation.

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GLOSSARY OF TERMS

Differentiation
A process by which a cell expresses specific gene products and adopts a shape required for functional specialization.

Gastrulation
The morphogenetic process by which cells of the blastula embryo undergo movements that establish the three-layered organization of the body plan.

Germ Layer
A major cell lineage that is established during gastrulation and that gives rise to ectodermal, mesodermal, and endodermal derivatives.

Induction
A process in which a group of cells produces an extracellular signal or set of signals that instructs the fate and differentiation of a distinct group of responsive cells.

Mesoderm
The primary germ layer that gives rise to bone, muscle (skeletal and heart), connective tissue, and the urogenital and circulatory systems.

Morphogenesis
A process of cell and tissue movements within the embryo that generates the appropriate shape of the body axis and of functional tissues and organs.

Specification
A state in which a cell has received the developmental inputs that instruct its future fate; however, it has not yet reached a state of determination.

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www.gastrulation.org
Jeff Hardin's Dynamics of Development:
worms.zoology.wisc.edu/embryology_main
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14 ENDODERM

DÉBORA SINNER, JAMES M. WELLS, and AARON M. ZORN

Division of Developmental Biology, Cincinnati Children's Research Foundation and Department of Pediatrics, School of Medicine, University of Cincinnati, Cincinnati, OH

INTRODUCTION

The endoderm, which is surrounded by the mesoderm and the ectoderm, is the innermost germ layer of the metazoan embryonic body plan (Figure 14.1). The term *endoderm* is derived from the Greek words *endo* and *dermis,* which mean "internal skin." The endoderm gives rise to the epithelial lining of the gastrointestinal and respiratory tracts and to the associated organs, such as the lungs, thymus, thyroid, liver, gallbladder, and pancreas.

One of the challenges of modern developmental biology is to understand, at the molecular level, how the primary germ layers are established. For a long time, research involving endoderm development lagged behind that of the mesoderm and the ectoderm, in part because of the difficulties associated with visualizing and experimentally manipulating this internal tissue. However, during the last 5 years, some of the key signaling molecules and transcription factors controlling endoderm formation in the frog Xenopus, the zebrafish, and the mouse have been described. Each of these animal models has its own experimental advantages. Xenopus embryos are easy to manipulate and allow for rapid analysis by the simple microinjection of recombinant RNA and DNA constructs. Zebrafish affords powerful forward genetic screens, and the analysis of the resulting mutants enables researchers to order the genes into coherent genetic pathways. Finally, gene targeting and embryonic stem cell technology in the mouse allows for the modeling of human development and disease. Comparing the results from these different model systems has accelerated our understanding of endoderm development and reveals that it is regulated by a conserved genetic pathway.

This chapter focuses on the molecular basis of vertebrate endoderm development, and it highlights how some of the genes involved are evolutionarily ancient and how they regulate endoderm development in various invertebrate species. Finally, we will summarize recent efforts to apply this newfound knowledge of endoderm formation to differentiate therapeutically useful endodermal tissues *in vitro* using human embryonic stem cells.

Principles of Developmental Genetics
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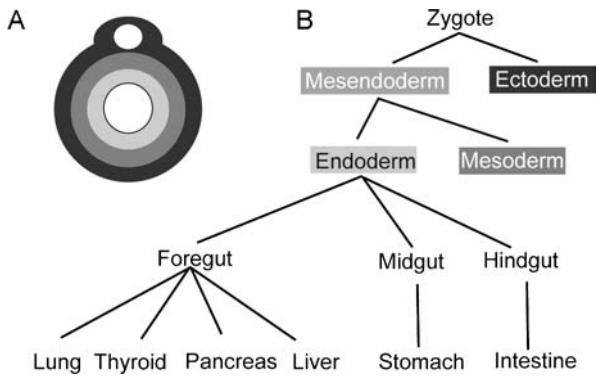

FIGURE 14.1 **The embryonic endoderm.** Endoderm, mesoderm, and ectoderm are the three primary germ layers of the vertebrate embryo. **A,** The schematic depicts a cross-section through a generalized vertebrate embryo after gastrulation, showing the relative positions of the ectoderm, the mesoderm, and the endoderm, which is the most internal layer. **B,** Development of the endoderm lineage. First, the ectoderm is segregated from the mesoderm and the endoderm, which are thought to have a common mesendoderm precursor. The mesoderm and the endoderm lineages are segregated during gastrulation. Later, during embryonic development, the endoderm is patterned along the anterior–posterior axis to give rise to the foregut, the midgut, and the hindgut, which ultimately contribute to the epithelium and the organs of the digestive and respiratory systems. (See color insert.)

I. OVERVIEW OF ENDODERM DEVELOPMENT

A. Fate Maps and Embryologic Experiments

Although the early embryos of different vertebrate species have divergent architectures and modes of morphogenesis, they all generate remarkably similar body plans after gastrulation and before organogenesis, when the endoderm forms a primitive gut tube. Initially, the gut tube is closed at both ends, but, later in development, it becomes perforated where the endoderm contacts the anterior and posterior ectoderm to form the mouth and the anus, respectively. The endodermal tube must also be patterned along the anterior–posterior and dorsal–ventral axes (see Chapter 40) through a complex series of reciprocal interactions between the endoderm and its surrounding mesenchyme. Eventually, organ primordia are induced, and they emerge as "outpockets" or organ buds from the gut tube (see the chapters in Section III, Morphogenetic and Cell Movements).

To understand the cellular and molecular mechanisms that control endoderm formation, it is essential to know from where in the early embryo the endoderm originates. This is done by labeling cells in living embryos and following what tissues those cells give rise to later in development. This process has allowed researchers to "fate map" the origin of the endoderm in Xenopus, zebrafish, and mice.

1. Xenopus

The large size and external development of the Xenopus embryo have led to a number of precise fate maps at different developmental stages. By microinjecting vital dyes into cells of the 32-cell embryo, researchers have determined that there is a gradation of germ layer fate along the animal–vegetal

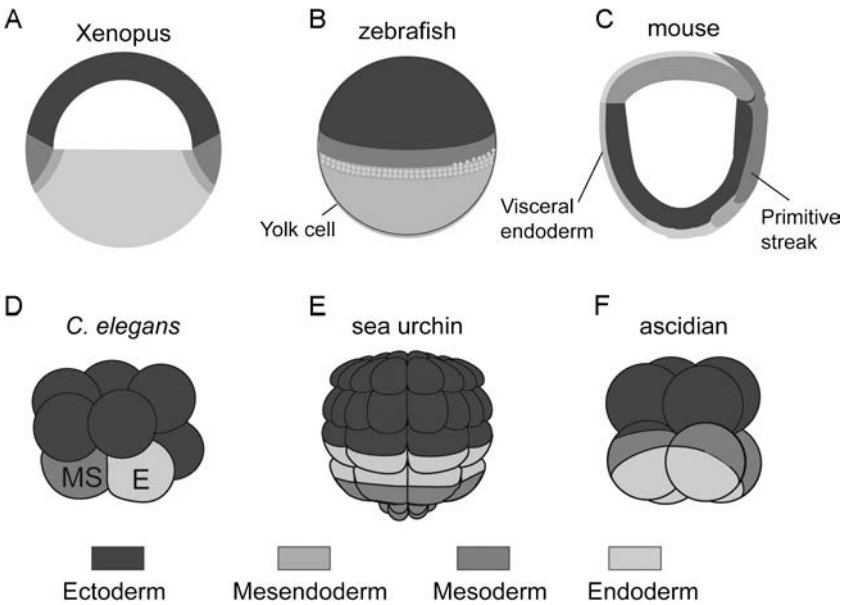

FIGURE 14.2 **Fate map of the endoderm in different species.** Embryonic fate maps of different species depicting the origins of the ectoderm (*blue*), the endoderm (*yellow*), the mesoderm (*red*), and the common mesendoderm precursors (*orange*). **A,** A Xenopus blastula is depicted in sections through the middle of the embryo, which shows that the endoderm originates from the yolky vegetal tissue, with mesendoderm and prospective mesoderm located at the equator. **B,** An external view of the zebrafish blastula shows the epiblast sitting on top of the large yolk cell. In the zebrafish, blastula endoderm and mesoderm precursors are intermingled in the marginal mesendoderm, with more endoderm cells coming from the future dorsal side (right). **C,** A cutaway view of the early mouse gastrula, with the definitive endoderm emerging from the anterior streak (bottom). Some of the primitive streak cells give rise to both endoderm and mesoderm; these are often referred to as *mesendoderm*. **D,** An 8-cell *C. elegans* embryo showing the MS and E cells that give rise to the mesoderm and the endoderm, respectively. These cells are derived from a single EMS, which is the mesendoderm cell at the 4-cell stage. **E,** A 64-cell sea urchin. **F,** An 8-cell ascidian embryo. (See color insert.)

(top–bottom) axis of the embryo. At the 32-cell stage, there are four tiers (rows) of cells, with the endoderm originating primarily from the two most vegetal (bottom) tiers and the ectoderm originating primarily from the two animal (top) tiers; mesoderm fate overlaps these, and it is primarily derived from the two middle tiers of cells (Dale and Slack, 1987; Moody, 1987). This general organization is maintained in the blastula and early gastrula, with the endoderm coming from the yolky vegetal cells, the mesoderm from a ring of equatorial tissue, and the ectoderm coming from the tissue on top of the blastocele cavity (Figure 14.2, A; Keller, 1976). Fate maps of neurula-stage embryos indicate that presumptive organ domains are arranged along the anterior–posterior axes in a pattern that is similar to their final position after the endoderm has elongated to form the gut tube (Chalmers and Slack, 2000).

In the blastula, vegetal cells induce the overlying equatorial region to form mesoderm (Nieuwkoop, 1969; see Chapter 13). During this period, the presumptive endoderm and mesoderm territories significantly overlap near the equator. Transplantation experiments indicate that cell fate is progressively determined and that vegetal cells are not committed to the endodermal lineage until the gastrula stage. When labeled single vegetal pole

cells were isolated from blastula or gastrula and transplanted into the blastoceles of host embryos, only the cells explanted from the gastrula stage contributed exclusively to endoderm-derived tissues of the host embryo (Wylie et al., 1987). More recent molecular analysis supports this timing of specification and indicates that, during the early blastula, equatorial cells express markers of both the endoderm and mesodermal lineages; however, by the mid-gastrula, there is a sharp boundary between these lineages (Wardle and Smith, 2004).

2. Zebrafish

In the zebrafish blastula, the epiblast, which will give rise to the embryo proper, sits on top of a large yolk cell. Fate-mapping experiments in the zebrafish blastula indicate that bipotential precursors of the endoderm and mesoderm (referred to as the *mesendoderm*) are localized to the marginal tissue next to the yolk cell. Most of the endoderm is derived from the two rows of cells closest to the yolk margin, predominantly from the dorsal lateral region, whereas the mesoderm precursors can be found up to eight cell diameters from the margin (Figure 14.2, B). The relative positions of presumptive organ domains in the endoderm correspond with the location of those organs along the anterior–posterior axis of the adult (Kimmel et al., 1990; Warga and Nusslein-Volhard, 1999). Transplantation experiments demonstrate that endodermal fate is progressively determined and that, as in Xenopus, cells are committed to the endoderm lineage at the onset of gastrulation (Warga and Nusslein-Volhard, 1999).

The first morphologically distinct endoderm cells in the zebrafish can be observed at gastrulation, when the marginal cells undergo an epithelial to mesenchymal transition and involute under the epiblast to form a hypoblast layer. The first marginal cells to involute are the endoderm cells, which flatten and extend filopodia to form a discontinuous cell layer next to the yolk cell. These cells now express endodermal marker genes *sox17* and *foxa2*. By contrast, the mesodermal cells of the hypoblast remain rounder and form a tissue layer between the endoderm and the noninvoluting epiblast, which becomes ectoderm (Warga and Nusslein-Volhard, 1999). After gastrulation and during the somatogenesis stages, the sheet of endoderm cells converges at the midline to form a solid endodermal rod, from which organ buds will later emerge (Ober et al., 2003).

3. Mouse

In the mouse embryo, two different types of endoderm are described: the *primitive* or *visceral endoderm,* which colonizes extraembryonic tissues, and the *definitive endoderm,* which contributes to fetal tissues. In this chapter, we are primarily referring to the definitive endoderm; however, the visceral endoderm sends important patterning signals to influence where the primitive streak and the mesendoderm will form during gastrulation (Yamamoto et al., 2004).

The pregastrula mouse embryo consists of a cup-like sheet of primitive epithelial cells known as the *epiblast,* which gives rise to all three germ layers; this is surrounded by extraembryonic visceral endoderm. Fate-mapping studies of prestreak embryos (embryonic days 6.0–6.5) indicate that the definitive endoderm originates from the posterior epiblast, where the primitive streak will form (Lawson and Pedersen, 1987; Tam et al., 2003). During early

gastrulation (embryonic days 6.5–7), endoderm precursors migrate through the anterior primitive streak and incorporate into the overlying visceral endoderm layer, eventually displacing the visceral endoderm into the extraembryonic region that becomes the yolk sac (Figure 14.2, C). The first definitive endoderm cells to emerge from the streak contribute to the foregut, whereas cells migrating through the streak later contribute to more posterior regions of the gut tube (Wells and Melton, 1999).

It is not entirely clear when endodermal fate is committed in the mouse (within the primitive streak or only after cells emerge from the streak), but fate-mapping studies support a progressive determination. Cells labeled before gastrulation in the region where the primitive streak will form contribute to all three germ layers, whereas cells labeled in the anterior streak after gastrulation has begun contribute to foregut and anterior mesoderm but not to ectoderm. The fact that the endodermal and mesoderm fates overlap substantially in the anterior streak supports the hypothesis that these cells are bipotential mesendoderm precursors (see Figure 14.2, C; Lawson and Pedersen, 1987; Tam et al., 2003). It is thought that, by the late gastrula, the endoderm cells are determined and can no longer become mesoderm or ectoderm. During somatogenesis stages, the anterior and posterior ends of the endoderm turn to produce foregut and hindgut pockets that eventually meet at the ventral midline, forming a gut tube.

B. Nodal Signaling

The Nodal-related family of transforming growth factor–beta (TGFβ) growth factors plays a key role in initiating mesendoderm development, and it is required for both the endoderm and mesoderm lineages in all vertebrate species examined. Nodal-related ligands act as morphogens, which are secreted diffusible factors that elicit distinct biological responses in cells, depending on the concentration and/or duration of exposure to the ligand. High levels of Nodal signaling promote endoderm fate, whereas lower levels induce mesoderm. As will be discussed in Section II, within the endoderm lineage, Nodal signaling controls the expression of a conserved group of transcription factors, including the Mix-like paired family of homeodomain proteins, Gata zinc finger factors, Sox HMG domain factors, and Fox forkhead domain factors, all of which regulate endoderm-specific gene expression and ultimately endoderm fate. Because of its central role in endoderm development, we will first introduce the major components of the Nodal signaling pathway (Figure 14.3; reviewed in detail in Schier, 2003).

Nodal was originally identified as a retroviral-induced mutation in mice (Conlon et al., 1994; Zhou et al., 1993), and, in addition to its role in mesendoderm development, Nodal signaling regulates many developmental processes, including left–right asymmetry and anterior–posterior patterning. Like other TGFβ family members, Nodal-related proteins are produced as preproprotein dimers in the secretory pathway; the release of mature ligands is regulated through proteolytic processing by proprotein convertases. In addition, Nodal signaling can be modulated in the extracellular space by secreted Nodal-antagonists such as Lefty, which binds to Nodal-related ligands, thereby preventing their interaction with the receptor complex. At the cell surface, Nodal-related ligands bind to a receptor complex that consists of two type II (ActRIIA or ActRIIB) and two type I (Alk4 or Alk7) transmembrane serine/

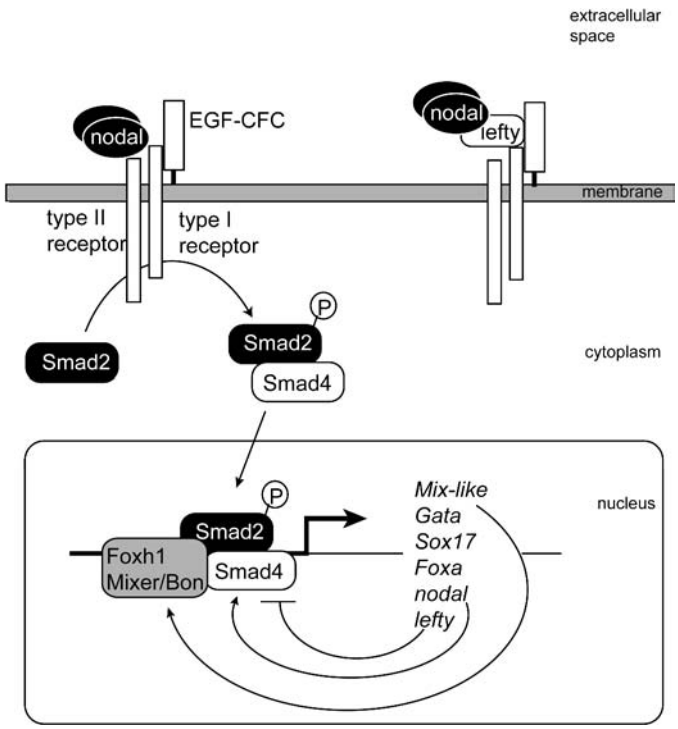

FIGURE 14.3 **The Nodal-signaling pathway.** The schematic shows the key proteins in the Nodal signaling pathway during vertebrate mesendoderm development. Nodal-related proteins are TGF-β ligands that are secreted as dimers. On the membrane of the receiving cell, Nodal ligands bind to the receptor complex, which contains type I and type II receptors and an EGF–CFC coreceptor. The activated receptor complex results in phosphorylation of the intracellular effector Smad2, which forms a complex with Smad4 and translocates to the nucleus, where it interacts with DNA-binding transcription factors such as Foxh1 to activate Nodal-responsive mesendoderm gene transcription. Among the transcriptional targets are *Nodal* genes themselves, and the Mix-like homeodomain factors, some of which also bind activated Smad2 and mediate Nodal signaling. These represent a feed-forward regulatory loop that propagates Nodal signaling. The secreted Nodal antagonist Lefty is also a direct transcriptional target, and it functions as a feedback inhibitor, because Lefty protein prevents the interaction of Nodal ligand with the receptor complex.

threonine kinases as well as an EGF-CFC family coreceptor (Cripto or Cryptic in mouse, Oep in zebrafish, and FRL1/XCR in Xenopus). After ligand binding, the activated type I receptor phosphorylates the cytosolic protein Smad2. Phosphorylated Smad2 binds the related protein Smad4, and this complex translocates to the nucleus, where it associates with DNA-binding transcription factors such as the Foxh1 (also known as FAST1) and some of the Mix-like factors to regulate mesendoderm gene transcription (see Figure 14.3).

II. MOLECULAR BASIS OF ENDODERM FORMATION IN VERTEBRATES

A. Xenopus

Xenopus mesendoderm development is initiated by the maternal T-box transcription factor, VegT, which is localized to the vegetal region of the oocyte and blastula. The depletion of maternal *VegT* mRNA by the microinjection of antisense oligos results in embryos lacking endoderm and that consist

almost entirely of ectoderm, with only residual amounts of mesoderm (Zhang et al., 1998). VegT is required for the zygotic transcription of most endoderm genes in the early blastula, including five Xenopus Nodal-related genes *(Xnr1, Xnr2, Xnr4, Xnr5,* and *Xnr6)* and a number of transcription factors, such as seven Mix-like genes, *Gata4, Gata5, Gata6,* and two *Sox17* genes, many of which are directly regulated by VegT DNA-binding sites in their promoters (Wardle and Smith, 2006; Xanthos et al., 2001). The vegetal tissue of VegT-depleted embryos is also impaired in its ability to induce mesoderm as a result of the loss of Nodal-related ligands. The injection of exogenous *Xnrs* back into VegT-depleted embryos can rescue endoderm and mesoderm development (Kofron et al., 1999; Xanthos et al., 2001), which indicates that one of VegT's primary roles is to initiate *Xnr* expression in the vegetal cells.

Analysis of the *Xnr1* and *Xn5* promoters reveals that they are directly regulated by a combination of maternal VegT and the Wnt/β-catenin pathway (Wardle and Smith, 2006). Maternal β-catenin promotes the higher expression of *Xnrs* on the dorsal side, which is important for anterior endoderm development. Thus, endoderm formation (via VegT) and early endoderm patterning (via β-catenin) are integrated at the transcriptional level. There is also evidence that two opposing maternal Sox transcription factors help restrict *Xnr5* expression to the vegetal region. Maternal Sox3 that is localized to the presumptive ectoderm appears to repress *Xnr5,* whereas maternal Sox7, which is enriched in the vegetal cells, binds the same DNA sites in the *Xnr5* promoter to stimulate transcription (Zhang et al., 2005).

TGFβ/Nodal signaling was first implicated in vertebrate mesendoderm development in *Xenopus,* where exogenous TGFβ2 and Activin were shown to cause naive blastula ectoderm to differentiate into ectopic mesoderm and endoderm tissue in a dose-dependent manner, with low doses inducing mesoderm and higher doses inducing endoderm (Hudson et al., 1997; Kimelman and Kirschner, 1987; Smith et al., 1990). Blocking endogenous TGFβ signaling by the overexpression of various dominant-negative molecules has also demonstrated that endoderm and mesoderm formation requires Nodal signaling in Xenopus (Agius et al., 2000; Henry et al., 1996; Osada and Wright, 1999).

By the late blastula, endoderm development becomes dependent on paracrine Nodal signaling between the vegetal cells. When blastula vegetal tissue is dissociated to disrupt cell signaling, endoderm gene expression is lost. However, this can be rescued in single, isolated vegetal cells that are overexpressing a constitutively active form of the Alk4 Nodal receptor (Clements et al., 1999; Yasuo and Lemaire, 1999), which indicates that Nodal signaling is sufficient to maintain endodermal transcription. Nodal signaling acts at several levels. First, it maintains *Xnr1* transcription via an enhancer that contains Foxh1/Smad DNA-binding sites (Osada et al., 2000), thus reinforcing high Nodal activity in the vegetal cells through an autoregulatory loop. Second, Nodal signaling maintains the expression of Mix-like, Gata4/5/6, and Sox17 transcription factors in the presumptive endoderm. Finally, Mixer, Bix2, and Bix3 interact with activated Smad2, which enhances their activity; thus, in addition to being Nodal targets, they are also effectors of Nodal signaling (Randall et al., 2002). This positive reinforcement of endoderm fate by Nodal signaling is balanced by secreted Nodal-antagonist Lefty. Lefty is a direct Nodal target in the mesendoderm, and it acts as a feedback inhibitor that prevents Nodal signaling from spreading into the ectodermal territory (Branford and Yost, 2002).

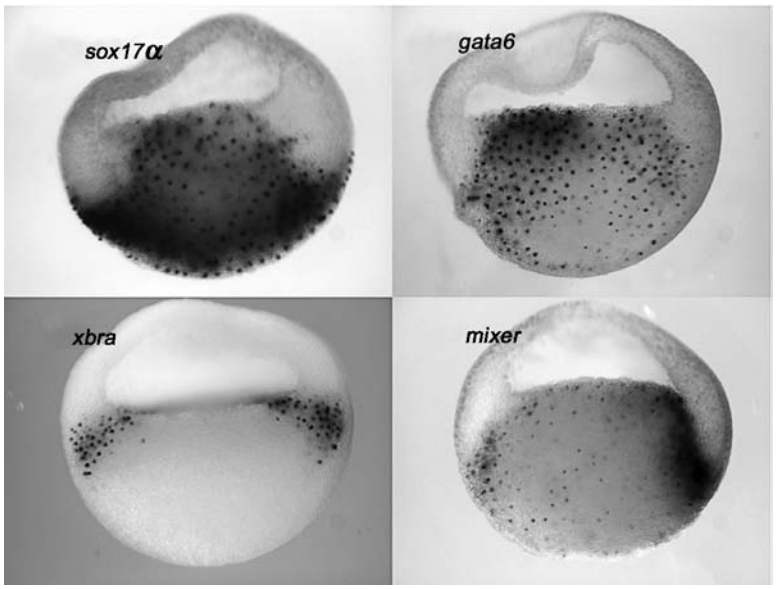

FIGURE 14.4 In situ hybridization to bisected Xenopus blastulae shows the expression of *Sox17α* and *Gata6* in the deep vegetal cells. *Mixer* transcripts are also expressed in the vegetal cells, but they are most abundant at the equator, overlapping with the expression of the mesodermal marker *Xbra*. (Images courtesy of Scott Rankin.)

Downstream of Nodal signaling, endoderm development is regulated by Sox17α, Sox17β, Gata4/5/6, and seven related Mix-like homeodomain proteins: Mix1, Mix2, Bix1/Mix4, Bix2/Milk, Bix3, Bix4, and Mixer/Mix3. In overexpression experiments, most of these can induce ectopic endoderm development and repress ectoderm and mesoderm fates. Sox17α/β and Gata4/5/6 are transcriptional activators that are expressed in the endoderm progenitors of the blastula and the early gastrula (Figure 14.4). Embryos depleted of both Sox17α and Sox17β have defects in endoderm formation, and development is often blocked during gastrulation (Clements et al., 2003; Hudson et al., 1997). The depletion of all three Gatas also disrupts endoderm formation resulting in gut tube defects and a loss of liver development (Afouda et al., 2005).

All seven Mix-like genes are transiently expressed in the blastula and gastrula vegetal cells, with particularly high expression levels seen in the equator at the future endoderm–mesoderm boundary (see Figure 14.4; Ecochard et al., 1998; Lemaire et al., 1998). To date, only Mixer has been examined in detail by loss-of-function experiments. In Mixer-depleted embryos, not only is endoderm development compromised, but many mesoderm genes are also upregulated, including *Fgf3* and *Fgf8* (Kofron et al., 2004; Sinner et al., 2006). Mixer-depleted vegetal tissue also had increased mesoderm-inducing activity (Kofron et al., 2004), probably as a result of the increased FGF levels, which can induce mesoderm but not endoderm (Cornell et al., 1995). Together with the enriched marginal expression, this data has led to the hypothesis that Mixer and probably other Mix-like transcription factors regulate the mesendoderm boundary by promoting endoderm development while at the same time repressing mesoderm fate.

The precise functional relationships between the Mix-like factors, Gata4/5/6, and Sox17 are unresolved, and, until recently, their downstream target genes were largely unknown. Initially a linear model was proposed in which Nodal signaling activated Mixer and Gata expression and they in turn promoted Sox17 expression, which then activated downstream endoderm target genes. However, recent microarray analysis has now identified hundreds of VegT, Nodal, Mixer, and Sox17 target genes (including Foxa transcription factors; Dickinson et al., 2006; Sinner et al., 2006; Taverner et al., 2005), and it has revealed that endoderm formation is much more complex than previously predicted. One study examined global endoderm gene expression in embryos in which Nodal signaling was blocked or in which either Mixer or Sox17 were depleted. Surprisingly, only 10% of endoderm genes behaved as predicted by the simple linear model. Rather, Nodal signaling, the Mix-like factors, Gata4/5/6, and Sox17 appear to interact in a complex network by reinforcing each others' expression as well as regulating overlapping and distinct downstream targets (Sinner et al., 2006).

There are ongoing efforts to generate interactive computer models to describe the complex gene regulatory networks controlling mesendoderm development (Loose and Patient, 2004). In the current model, the combined action of these transcription factors commits vegetal cells to the endodermal fate by activating the appropriate endoderm transcriptional program downstream of nodal signaling while at the same time repressing the mesodermal program (Figure 14.5, A).

B. Zebrafish

Large-scale genetic screens in zebrafish have independently identified a remarkably similar set of genes controlling endoderm formation, including components of the Nodal pathway, *gata5*, two Mix-like homeobox genes *(bonnie and clyde [bon]* and *mezzo)*, and two Sox genes *(casanova [cas]* and *sox17)*. Sophisticated genetic analyses coupled with overexpression studies have provided a powerful approach to establishing a model of the functional hierarchy between these genes in zebrafish (Stainier, 2002).

In the current model (see Figure 14.5, B), mesendoderm development is initiated in the marginal region of the early blastula by an unknown signal from the underlying yolk syncytial layer (YSL), an extraembryonic structure that is associated with the yolk cell (Ober et al., 2003; Stainier, 2002). Zebrafish do not appear to use a maternal T-box transcription factor that is equivalent to Xenopus VegT. The YSL signal induces the expression of the two Nodal related genes *squint* (*sqt*) and *cyclops (cyc)* in the two or three cell tiers closest to the yolk, with higher expression on the dorsal side as a result of Wnt/β-catenin signaling (Schier, 2003).

Nodal signaling is absolutely necessary and sufficient to activate the cascade of mesendoderm development. *Sqt* and *cyc* are partially redundant. However, embryos in which both *cyc* and *sqt* are mutated completely lack endoderm and the majority of mesoderm tissue; this phenotype is similar to *one-eyed pinhead* mutants *(MZoep)* that lack both the maternal and zygotic contributions of the EGF–CFC Nodal coreceptor and that are devoid of Nodal signaling (Ober et al., 2003; Schier, 2003). The overexpression of a constitutively active Nodal receptor is sufficient to rescue endoderm in *MZoep* mutants and to induce ectopic mesendoderm throughout the epiblast, independent of an inducing signal from the YSL (Ober et al., 2003; Schier,

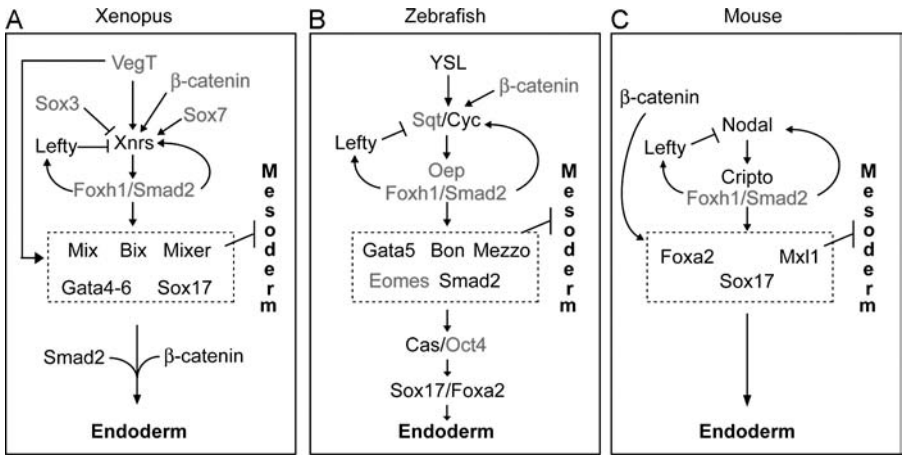

FIGURE 14.5 A model of the molecular pathway that regulates endoderm formation in Xenopus, zebrafish, and the mouse. Gray text indicates maternally supplied gene products. **A,** In Xenopus, the maternal T-box transcription factor VegT (along with β-catenin and Sox7) initiates mesendoderm development and activates the zygotic expression of the Xnr, Nodal-related ligands, and the transcription factors Sox17, Mix1/2, Bix1/2/3/4, Mixer, and Gata4/5/6. During the early blastula, high levels of Nodal/Foxh1/Smad2 signaling activity maintain their expression. Nodal signaling also maintains *Xnr* expression in an autoregulatory loop, and the nodal antagonist Lefty keeps this in check. In the vegetal cells of the blastula, Sox17α/β, Gata4/5/6, and the Mix-like factors promote each other's expression in a complex regulatory network. These transcription factors have both common and distinct sets of downstream target genes. Sox17 and Mixer interact with β-catenin and Smad2, respectively, to regulate some of their downstream targets. The Mix-like genes also appear to repress some mesoderm gene expression. **B,** In zebrafish, an unknown signal from the yolk syncytial layer initiates mesendoderm development and the expression of Nodal-related ligands *sqt* and *cyc* at the margin. High levels of Nodal signaling mediated by the Oep coreceptor and Foxh1/Smad complexes activates the expression of Gata5, Bon, and Mezzo in the mesendoderm. Nodal signaling is maintained by autoregulation and feedback inhibition by Lefty. *Gata5*, Bon, and Mezzo are partially redundant, and they act in parallel to promote endoderm fate as well as to repress mesoderm gene expression. In the marginal tissue Gata5, Bon, and Smad2 form a complex with the maternally localized T-box transcription factor Eomes to promote *Cas* transcription in the endodermal precursors. Cas, acting with maternal Oct4, regulates the expression of endodermal genes Sox17 and Foxa2. **C,** In the mouse, high levels of nodal signaling in the anterior primitive streak initiate definitive endoderm development, which is mediated by the nodal coreceptor Cripto and Foxh1/Smad2 transcriptional complexes. Nodal signaling is maintained by autoregulation and feedback inhibition by Lefty. Downstream of Nodal signaling, the transcription factors Mixl1, Foxa2, and Sox17 are expressed in the endoderm and mesendoderm progenitor cells that are emerging from the streak. Each of these transcription factors has a slightly different expression pattern, and each regulates distinct aspects of endoderm development. Mixl1 appears to function in part by repressing some mesoderm gene expression, whereas Foxa2 and Sox17 are necessary for anterior and posterior endoderm, respectively.

2003). Biochemical and genetic experiments that progressively reduce the levels of Nodal signaling result in the loss of endoderm tissue before mesoderm, which indicates that, in zebrafish, Nodal signaling acts in a dose-dependent manner. Nodal activity is highest in the first two rows of marginal cells, which express *cyc* and *sqt* and which are fated to give rise to endoderm, whereas lower doses of secreted Sqt induce mesodermal fate in up to eight to ten rows of cells from the margin. The range of Nodal activity is regulated by the balance between Sqt and Cyc promoting their own expression and feedback repression by the Nodal antagonist Lefty (Schier, 2003).

In addition to the dose of Nodal, other mechanisms must also regulate the allocation of the endoderm and mesoderm lineages, which are intermingled in the margin. Candidates include the notch–lateral inhibition pathway and the fibroblast growth factor (FGF) pathway, which promote mesoderm but not endodermal fate (Kikuchi et al., 2004; Rodaway et al., 1999); however, their precise roles have not been determined.

In response to Nodal signaling, Foxh1/Smad2 complexes are required to activate the expression of *cas,* gata5, *bon*, and *mezzo* in overlapping domains in the mesendoderm (Kunwar et al., 2003). *Cas* is expressed in the two tiers of cells next to the margin, and it is thought to mark the endoderm progenitors. *Gata5* is expressed up to five cells from the margin, whereas the Mix-like genes *bon* and *mezzo* are expressed in a broader band that is approximately eight cells wide and that contains both endoderm and mesoderm precursors (Ober et al., 2003).

Analyses of mutant embryos indicate that these zygotic transcription factors specify the endoderm fate from the bipotential mesendoderm precursors during the late blastula. Mutations in the gata5 locus, known as *faust,* have approximately 50% fewer endoderm cells at the gastrula stage, and the remaining endoderm cells have lower expression levels of the endodermal markers *sox17* and *foxa2* (Reiter et al., 2001). *Bon* mutants exhibit a 70% to 90% reduction in the number of endoderm cells at gastrulation, and, later, the gut tube is almost entirely absent (Kikuchi et al., 2000). Although mutations in *mezzo* are not reported, experiments with antisense oligos indicate that it is partially redundant with *bon*. In overexpression assays, *bon* and *mezzo* can both induce ectopic endoderm in the margin while repressing mesoderm fate (Poulain and Lepage, 2002).

Epistasis experiments indicate that *mezzo, bon,* and gata5 act in parallel, downstream of Nodal signaling and upstream of *cas* (Ober et al., 2003). Genetic and biochemical studies suggest that, in the marginal cells, Smad2, Bon, and Gata5 physically interact with the maternal T-box transcription factor eomesodermin (which is localized to the marginal region), resulting in a complex that may directly activate *cas* transcription (Bjornson et al., 2005). In the future, it will be important to determine if eomesodermin in Xenopus and mouse (which so far have only been implicated in mesoderm development) also have roles in endoderm formation in those species.

The divergent Sox protein Cas is cell-autonomously required for endoderm development downstream of *bon, mezzo,* and gata5 (Ober et al., 2003). *Cas* mutants completely lack endoderm precursors at the onset of gastrulation, and they do not form a gut tube (Alexander et al., 1999). Unlike *bon, faust,* and mutations in the Nodal pathway, which also have mesodermal defects, *cas* function appears to be endoderm specific. Genetic and biochemical experiments indicate that *cas* cooperates with the ubiquitously expressed Pou-domain transcription factor Oct4 (also known as *spg, pou2,* and *pou5f1*), and they may physically interact to activate the transcription of *sox17* and *foxa2* (Lunde et al., 2004; Reim et al., 2004). At gastrulation, *sox17* and *foxa2* are expressed in the specified endodermal cells after they involute into the hypoblast. Although the functions of Sox17 and Foxa2 have not been determined in zebrafish, the model predicts that they regulate the transcription of downstream genes required for subsequent endoderm differentiation.

C. Mouse

Germ layer formation in mouse embryos is inextricably linked to gastrulation, because the endoderm and mesoderm arise from the primitive streak. Before gastrulation, the anterior visceral endoderm secretes Nodal antagonists Lefty and Cer1, which are thought to limit Nodal and Wnt signaling activity to the prospective posterior epiblast, thus establishing the site where gastrulation and endoderm development will be initiated (Yamamoto et al., 2004). Despite the difference in how the mouse, frog, and fish initiate endoderm formation, many of the same molecules found in frogs and fish also operate in the mouse.

In the mouse, Nodal is required for the formation of the primitive streak (Conlon et al., 1994; Zhou et al., 1993) and for subsequent mesendoderm development, as are various components of the downstream signaling pathway. Gene knockouts of the Nodal coreceptor Cripto, the intracellular effector Smad2, and its binding partner Foxh1 all result in gastrulation, endoderm-specification defects, or both (Ding et al., 1998; Hoodless et al., 2001; Waldrip et al., 1998). Smad2 and FoxH1 are required for definitive endoderm development. In chimeric studies in which mutant embryonic stem cells are added to wild-type embryos, cells lacking *Smad2* rarely contribute to any regions of the developing gut tube. Interestingly, embryonic stem cells lacking *Foxh1* fail to contribute to the foregut, but they populate to the hindgut, suggesting that other Smad2 interacting proteins must regulate the Nodal-dependent endoderm development of the posterior endoderm.

Genetic experiments to reduce the levels of Nodal signaling, either with hypomorphic *Nodal* alleles or by ablating *Smad2* specifically in the epiblast (Tam et al., 2003), indicate that Nodal regulates mesendoderm fate in a dose-dependent manner, with high Nodal activity required for endoderm specification. Analysis of the regulatory elements controlling *Nodal* transcription indicate that, as in Xenopus and zebrafish, Nodal maintains its own expression in an auto-regulatory loop. *In vivo* ablation of the Foxh1 binding site in that enhancer results in the disruption of Nodal expression and defects in definitive endoderm specification (Norris et al., 2002).

Canonical Wnt/β-catenin signaling has also been implicated in the allocation of endoderm versus mesoderm lineages in the mouse. Conditional knockout of *β-catenin* in the epiblast results in defects in definitive endoderm formation and ectopic cardiac mesoderm, which could be the result of the impaired endoderm differentiation of a mesendoderm progenitor cell (Lickert et al., 2002).

Gene knockout studies and chimeric analyses indicate that the transcription factors Sox17, Mixl1, and Foxa2 regulate endoderm formation downstream of Nodal and Wnt/β-catenin. In contrast with fish and frogs, Gata4/5/6 appear to not be involved in definitive endoderm formation in the mouse but rather to regulate extraembryonic endoderm development and later aspects of endoderm organ formation (Zhao et al., 2005).

Foxa2 is expressed in the visceral endoderm, the primitive streak, the notochord, the floor plate, and the anterior definitive endoderm, and embryos lacking *Foxa2* have severe defects in foregut and midgut but not hindgut development (Dufort et al., 1998). In the mouse, *Sox17* transcripts are first observed in the visceral endoderm and then in the definitive endoderm emerging from the anterior primitive streak; however, by embryonic day 8, *Sox17* is restricted to the hindgut. *Sox17*-deficient embryos have a relatively

late endoderm phenotype, with severe hindgut defects only after gastrulation; the foregut is relatively unaffected (Kanai-Azuma et al., 2002).

In the mouse, there is only one Mix-like gene *(Mixl1)*, and it is expressed in the primitive streak at the time of endoderm formation (Pearce and Evans, 1999). Evidence from tissue culture experiments suggests that, similar to Xenopus and zebrafish Mix-like genes, *Mixl1* transcription is activated by Nodal signaling via Foxh1/Smad DNA-binding sites in its promoter (Hart et al., 2005). It is not clear whether the Mixl1 protein interacts with Smad2 to regulate Nodal-target genes like Mixer or Bon do, because Mixl1 does not contain an obvious Smad2-binding motif (Randall et al., 2002). Embryos lacking *Mixl1* do not have an overt endoderm specification defect, and mutant $Mixl1^{-/-}$ embryonic stem cells contribute to most endoderm tissues, except the hindgut (Hart et al., 2002). However, $Mixl1^{-/-}$ embryos exhibit a thickened anterior primitive streak with excessive growth in axial mesoderm tissue (Hart et al., 2002); this suggests that, like Mixer and Bon, Mixl1 might function in part by repressing mesoderm development.

Although many of the hierarchical relationships between the genes regulating mouse endoderm development have not been directly tested in the mouse, there are some clear similarities between the genetic pathways in Xenopus and zebrafish (see Figure 14.5, C). One important observation emerging from these mouse studies is that Foxh1, Foxa2, Sox17, and Mixl1 regulate discrete downstream endodermal lineages, suggesting that they have distinct target genes.

III. MOLECULAR BASIS OF ENDODERM DEVELOPMENT IN INVERTEBRATES

Endoderm development has also been extensively studied in a number of invertebrate and lower chordates species, including sea urchins, *Caenorhabditis elegans,* and ascidians. These model organisms have some powerful experimental advantages, and they provide important insight into the evolutionarily conserved genetic program of endoderm development. In all of these species, germ layer development is stereotypical and rather invariant, allowing for the unambiguous identification of all of the endoderm progenitors cells during the early cleavage stage. In the sea urchin, there is a long history of experimental embryology, and exquisite cell transplantation experiments are possible. *C. elegans* has powerful genetics and a loss of gene function by RNAi; in ascidians, it is very easy to generate transgenic embryos by simple electroporation. Thus, in each of these systems, the endoderm progenitor cells can be experimentally manipulated in a very elegant and precise manner. The results from these organisms have revealed some ancient conservation in the genes controlling endoderm development as well as some important distinctions with higher vertebrates.

A. *Caenorhabditis elegans*

In the nematode *C. elegans,* a single mesendoderm precursor cell (EMS) can be identified in the 4-cell embryo. When EMS divides, one of its daughter cells, known as *MS,* gives rise to mesoderm lineage, whereas the other daughter, known as the *E cell,* gives rise to the endoderm (Figure 14.2, D; Maduro and Rothman, 2002). The regulation of E fate is controlled by two cooperating

maternal signals. The first is the maternal homeodomain transcription factor SKN-1, which initiates the development of the common mesendoderm progenitor by activating the transcription of two Gata genes, *med-1* and *med-2,* in EMS. In the MS lineage, MED-1 and MED-2 proteins promote mesoderm gene expression, whereas, in the E cell, they regulate the expression of two other Gata genes, *end-1* and *end-2,* which are required for endoderm development (Maduro and Rothman, 2002).

The second maternal input is a Wnt signal that regulates whether mesoderm- or endoderm-specific genes are activated downstream of MEDs. As EMS divides, the E cell receives a signal from the adjacent P2-cell, thus activating a Wnt signal transduction pathway that induces the endodermal program. In the MS cell, which does not receive the Wnt signal, the TCF/LEF type transcription factor POP-1 represses *end-1* and *end-3* expression, thus inhibiting endodermal fate. In the E cell, Wnt signaling appears to switch the activity of POP-1 from a transcriptional repressor to an activator, which cooperates with the MED factors to stimulate *end-1* and *end-2* transcription (Shetty et al., 2005). Within the endodermal lineage, END-1 and END-2 activate the expression of two more Gata genes, *elt-2* and *elt-7,* which function redundantly to regulate the expression of many genes in the developing gut (Maduro and Rothman, 2002), including a FoxA-like gene called *pha-4* that regulates foregut development.

B. Sea Urchin

In sea urchin embryos, mesendoderm development is initiated by an unidentified signal that causes an accumulation of nuclear β-catenin in the vegetal nuclei by the 16-cell stage. Nuclear β-catenin/TCF complexes are required to activate a cascade of downstream mesendoderm factors, such as Wnt8, and several transcription factors, including Krox, Krl, and the paired homeodomain repressor Pmar1 (the same general class of proteins as the Mix-like factors; Oliveri and Davidson, 2004). These in turn activate downstream mesendoderm genes, and they promote the clearance of SoxB1 and SoxB2 proteins from the vegetal nuclei, which, like Sox3 in Xenopus would otherwise promote ectoderm fate at the expense of the mesendoderm (Kenny et al., 2003; Oliveri and Davidson, 2004). The segregation of the endoderm and mesoderm lineages is regulated by Notch signaling. Mesendoderm cells in which the Notch pathway is activated become mesoderm, whereas those in which Notch is not activated become endoderm (Figure 14.2, E). Within the endodermal lineage, the transcription factor GataE is expressed, and this regulates most of the downstream endoderm genes, including other transcription factors, such as FoxA (Oliveri and Davidson, 2004).

Recently, Davidson et al. (2002) have integrated the results from many laboratories, including experimental perturbations, large-scale genomic information, and detailed analysis of the regulatory transcription factors and the DNA-binding sites in their target gene promoters to assembled a gene regulatory network (GRN), which is a computer model that describes the logic of the many complex interactions and transcriptional inputs that control mesendoderm development (Davidson et al., 2002; Oliveri and Davidson, 2004). The success of this approach has prompted investigators in other model system to generate similar GRNs, such as the emerging mesendoderm GRN for Xenopus (Loose and Patient, 2004).

C. Ascidians

Ascidians are urochordates, a basal group of chordates that have a notochord and a larva body plan that is similar to that of higher vertebrates. Classic embryologic experiments have precisely mapped all of the cell lineages with in the ascidian embryos. As early as the 8-cell stage, it is possible to distinguish portions of the blastomeres that give rise to the endoderm (Figure 14.2, F); at the 32-cell stage, four vegetal endoderm progenitor cells can be identified. Similar to that seen in sea urchin, mesendoderm development in ascidians appears to be initiated by the nuclear accumulation of maternal β-catenin in the endoderm progenitors at the cleavage stage. β-Catenin/Tcf complexes regulate the expression of several zygotic transcription factors involved in endoderm formation, including the homeodomain factor TTF1, the LIM-homeobox Lhx3, and the forkhead factor FoxA5, which together regulate downstream endoderm differentiation genes (Nishida, 2005).

IV. CONSERVED MOLECULAR MECHANISMS

A. Interspecies Comparisons

A comparison of the genes controlling endoderm formation between various species reveals some striking conservation. Clearly, the Gata, FoxA, and paired homeodomain transcription factors constitute an evolutionarily ancient genetic cassette (with the exception of Gata in the mouse). It is also interesting to note that Wnt/β-catenin signaling is involved in all species; however, its relative importance has changed over evolution. Although β-catenin is the central player in the initiation of mesendoderm development in invertebrates, it appears to have a more peripheral role in vertebrates, and it is more involved in endoderm patterning. In addition, the SoxF factors such as Sox17 and Casanova, which are central to vertebrate endoderm development, do not appear to be involved in invertebrates. The most striking difference between vertebrates and lower species is the central role of nodal signaling. Although Nodal-related genes have been found in deuterostome genomes, including sea urchin and ascidians, there is no evidence that they are involved in mesendoderm formation in those species.

Although each vertebrate species appears to use a different mechanism to initiate endoderm development, in each case, Nodal signaling is at the top of the genetic hierarchy controlling the expression of Mix-like, SoxF, FoxA, and Gata transcription factors. Together, these factors make up a core regulatory circuit, which Xenopus, zebrafish, and mice use in slightly different ways to promote endoderm development.

B. Separating Endoderm from Mesoderm

On the basis of fate-mapping studies and experimental evidence from the different species reviewed in this chapter, the concept that the endoderm and mesoderm lineages have a common mesendoderm progenitor has recently gained momentum (Rodaway and Patient, 2001). Therefore, a critical role of the core endoderm regulatory genes is to control the segregation of the endoderm and mesoderm lineages from the mesendoderm progenitor, and it appears that a similar set of molecular mechanisms regulates this process in

vertebrates. Next is presented a summary of a generalized model that encompasses data from Xenopus, zebrafish, and mice.

Nodal signaling is the first critical event, with low doses promoting mesoderm and high doses required for endoderm. In Xenopus and zebrafish, the endoderm forms predominantly in cells that produce the Nodal-related ligands, whereas mesoderm is induced in adjacent tissue, which receives lower Nodal levels. Early in development, some mesendoderm progenitors express both mesoderm and endoderm genes, but these expression domains become mutually exclusive by the mid gastrula (Wardle and Smith, 2004). This appears to be controlled by paracrine Nodal signaling between cells in the presumptive endoderm territory, which sets in motion a positive feedback loop that amplifies Nodal signaling and rapidly establishes the endoderm transcription profile at the exclusion of the mesoderm program. First, Foxh1/Smad2 complexes enhance the expression of Nodal-related ligands. Second, Nodal signaling maintains the expression of Mix-like, Gata, SoxF, and FoxA transcription factors, and these promote endoderm gene expression. Finally, activated Smad2 enhances the activity of some of the Mix-like factors.

The Mix-like proteins appear to repress mesoderm gene expression while at the same time promoting endoderm fate. At the biochemical level, it is unclear how they do this, but evidence from Xenopus suggests that Mixer acts in part by restricting the expression of FGF ligands to the presumptive mesoderm and excluding them from the endoderm. In Xenopus and zebrafish, FGF signaling has been shown to act downstream of Nodal to promote mesoderm but not endoderm fate. Thus, FGF signaling in the presumptive mesoderm territory may suppress the endoderm program while at the same time promoting mesoderm gene expression.

Other signaling pathways may also be involved, such as Notch and Wnt, which regulate the segregation of endoderm and mesoderm progenitors in invertebrates. However, more work is required to rigorously test the role of these pathways. At the moment, this generalized model is an amalgamation of results from Xenopus, fish, and mice. It will be important to test the details in each species, because there are clearly some differences among the vertebrates. Some important unresolved questions still remain. For example, it is not clear how different doses of Nodal signaling and, hence, different amounts of phosphorylated Smad2 can regulate endoderm versus mesoderm genes. The evidence suggests that Foxh1 and Mixer/Bon have overlapping target genes, but this cannot account for all Nodal-dependent mesendoderm gene expression. Further work is also needed to resolve the relationships between the endoderm transcription factors and their downstream targets. However, even with these limitations, this model has already proven useful to researchers trying to produce endodermal tissues from human stem cells.

V. CLINICAL RELEVANCE: MAKING ENDODERMAL TISSUE FROM STEM CELLS

A large number of severe human diseases affect endodermally derived cell types. For example, defects in pancreas development can result in severe chronic pancreatitis, with the ultimate failure of both digestive and hormonal functions, and failed bile duct development can result in lethal biliary atresia. In addition, the endoderm sends essential inducing signals to various mesodermal tissues, including the heart and the head. Studies of later endoderm organ

development in model organisms have identified the underlying mechanisms of many congenital disorders in humans. By contrast, major defects in endoderm specification are likely to result in early embryonic lethality and will probably not be observed in the clinic. However, an understanding of early endoderm formation is having an important impact on the burgeoning field of regenerative medicine, where researchers are attempting to make therapeutically useful tissue from stem cells (Kume, 2005).

Initial attempts at differentiating embryonic stem cells directly into mature endoderm derivatives, such as liver hepatocytes and pancreatic β-cells, gave only moderate success (Kume, 2005). More recently, the view has emerged that embryonic stem cells probably must follow a developmental progression similar to normal embryogenesis by first forming definitive endoderm before being competent to differentiate into organ-specific cell types. Several groups have now used paradigms based on the current understanding of endoderm development to efficiently direct the differentiation of mouse and human embryonic stem cell cultures into definitive endoderm-like cells by activating the Nodal pathway with recombinant Activin (a Nodal-like TGFβ ligand; D'Amour et al., 2005; Kubo et al., 2004; Tada et al., 2005; Yasunaga et al., 2005). Remarkably, the concentration-dependent effects of Activin (and Nodal) on embryonic stem cell differentiation are nearly identical to its mesendoderm-inducing activity on naive ectoderm cells (animal caps) in frog, with low Activin concentrations generating mesoderm and higher concentrations promoting endoderm. The molecular response of embryonic stem cells is also very similar to the kinetics of normal development. One day after the treatment of embryonic stem cell cultures with Activin, they initiate mesendoderm gene expression that is indicative of early gastrulation. After 2 to 3 days, the cultures express mid-gastrulation markers such as *Mixl1,* and, after 3 to 5 days, the cells express definitive endoderm markers, including *Sox17* and *Foxa* (D'Amour et al., 2005; Kubo et al., 2004; Tada et al., 2005; Yasunaga et al., 2005). Overall, this suggests that the Activin-treated embryonic stem cells pass through a mesendoderm precursor before a subset of them select either an endoderm or mesoderm lineage.

The use of Activin to promote the efficient differentiation of human embryonic stem cells into the definitive endoderm lineage demonstrates the importance of developmental biology in model organisms. The hope is that these endoderm cells will be a renewable source of material that can be efficiently differentiated into therapeutically important lineages, such as pancreatic β cells, to treat diabetes. Undoubtedly, the realization of this goal will continue to rely on information obtained from studies in frogs, fish, and mice.

SUMMARY

- Comparing and contrasting results from different animal modes has accelerated our understanding of the molecular program that controls endoderm development.
- Evidence increasingly supports the hypothesis that the endoderm and mesoderm are derived from a common mesendoderm progenitor and that these lineages are segregated during gastrulation.

- Although amphibian, fish, and mammalian embryos use distinct cues to initiate germ layer formation, in each case, these engage a conserved Nodal-signaling pathway to activate mesendoderm development.
- Differential Nodal signaling is a key event in separating the vertebrate endoderm and mesoderm lineages. Within the endoderm lineage, high levels of Nodal signaling activate a conserved regulatory circuit consisting of Mix-like, Sox, Fox, and Gata transcription factors. Each species appears to use these factors in similar but slightly different ways to promote endoderm development while repressing the mesoderm program.
- Some of the components of this pathway, such as Gata and FoxA factors, are evolutionarily ancient. By contrast, Nodal signaling appears to be vertebrate specific. Wnt/β-catenin signaling is the key event that initiates mesendoderm development in invertebrates; however, in vertebrates, this is more important during early endoderm pattering.
- Understanding the molecular basis of endoderm development in these animal models is clinically significant, because researchers have successfully begun to use these developmental paradigms to direct the development of endoderm tissue from human embryonic stem cells. Ultimately, this work has the promise of producing a renewable source of material for cell-based therapies for many life-threatening diseases that affect endodermal organs.

ACKNOWLEDGMENTS

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GLOSSARY OF TERMS

Endoderm
The innermost embryonic germ layer that gives rise to the lining of the gastrointestinal and respiratory tracts and their associated organs.

Gene regulatory network
A logical map that integrates transcription factors and regulatory elements by controlling the expression of these genes.

Mesendoderm
A progenitor cell population that gives rise to both endoderm or mesoderm lineages.

Visceral endoderm
A tissue in the mammalian embryo that gives rise to the extraembryonic membranes. This tissue does not contribute to the embryo proper, but it is an important source of signals for the formation of primitive streak and mesendoderm.

Yolk syncytial layer
An extraembryonic structure on the surface of the yolk cell in the fish embryo that is an important source of inductive signals to mesendoderm.

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RECOMMENDED WEB SITES

The Sea Urchin Mesendoderm Gene Regulatory Network:
http://sugp.caltech.edu/endomes/
The Xenopus Mesendoderm Gene Regulatory Network:
http://www.nottingham.ac.uk/genetics/networks

15

NOTCH SIGNALING: A VERSATILE TOOL FOR THE FINE PATTERNING OF CELL FATE IN DEVELOPMENT

AJAY B. CHITNIS

Section on Neural Developmental Dynamics, Laboratory of Molecular Genetics, Bethesda, MD

INTRODUCTION

The Notch signaling pathway is an evolutionarily conserved signaling pathway that allows a cell to communicate with its neighbor and regulate its fate (Lai, 2004). In doing so, it plays extraordinarily diverse roles in defining how different types of cells are distributed within a tissue. By regulating the spatiotemporal pattern of differentiation and cell morphology, this signaling pathway can determine the number of progenitor cells and regulate the acquisition of distinct fates or morphologic features within a particular tissue. The goal of this chapter is to illustrate how the integration of local interactions mediated by Notch signaling with other signaling mechanisms facilitates the fine patterning of cell fate within a tissue. The examples selected will highlight the important role of protein trafficking in the regulation of the efficacy of both ligand and receptor function in Notch signaling. The chapter will illustrate how the modulation of trafficking by additional signaling mechanisms can bias the outcome of Notch signaling and reliably determine the pattern of cell differentiation at the single-cell level.

A. The Notch Signaling Pathway

At the core of the signaling pathway is the Notch family of single-pass transmembrane proteins, which have extracellular domains that are characterized by a variable number of epidermal growth factor (EGF)–like repeats (Figure 15.1; Lai, 2004). It mediates interactions with Delta Serrate Lag-2 (DSL) family members that are also single-pass transmembrane proteins with extracellular EGF-like repeats but that have, in addition, a conserved

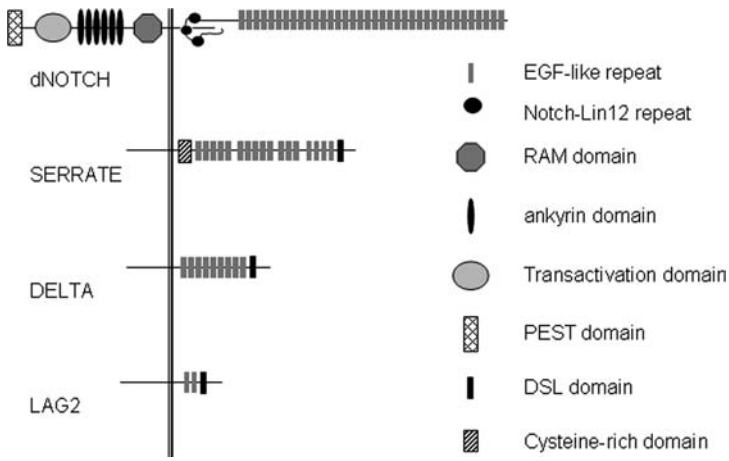

FIGURE 15.1 The structure of Notch family receptors and Delta Serrate Lag-2 ligands. The structure of Drosophila Notch, Serrate, and Delta and *Caenorhabditis elegans* Lag2 are shown as examples. The Notch–Lin-12 family of receptors has a variable number of epidermal growth-factor–like repeats (36 in dNotch1) and three Notch–Lin-12 repeats in the extracellular domain, an intracellular RAM domain, an ankyrin repeat domain, a transactivation domain, and a PEST domain. The RAM domain initiates interactions with the CBF1/Su(H)/Lag1 (CSL) protein, and this is followed by an additional interaction between CSL and the ankyrin repeat domain, which creates a surface for Mastermind interaction. The interaction of the RAM and ankyrin repeat domains with CSL produces a conformational change in CSL that might convert it from a repressor to an activator of Notch target genes (Nam 2006; Wilson 2006). The PEST domain facilitates degradation of the Notch intracellular domain fragment in the nucleus by the E3 ligase Sel10 (Oberg, 2001).

N-terminal DSL domain (Fleming, 1998). The Notch extracellular domain (NECD) is cleaved in a Furin-dependent manner from the rest of the Notch protein before it becomes functionally active on the cell surface; however, it remains bound noncovalently in a calcium-dependent manner to the remaining extracellular stub (Figure 15.2; Logeat et al., 1998). The Furin-dependent cleavage (S1) is the first of three cleavage events (S1, S2, and S3) that are critical for the eventual activation of Notch. A key step in activation of the Notch family of receptors is the removal of the extracellular domain (ECD). Interaction of the NECD with the ECD of a DSL ligand expressed on the surface of a neighboring cell facilitates both the separation of the ECD from the rest of receptor and the S2 and S3 proteolytic cleavages that eventually release the Notch intracellular domain from the cell surface into the cell. The S2 cleavage is in the Notch extracellular stub, and it is mediated by specific ADAM (A disintegrin and metalloproteinase domain) proteases (Mumm et al., 2000). The membrane-tethered Notch fragment left after S2 cleavage is referred to as the *NEXT* fragment, and it is a constitutive substrate for the S3 cleavage. The S3 cleavage is an intramembranous event, and it is mediated by the multimeric γ-secretase complex (De Strooper et al., 1999), which includes a Presenilin heterodimer, Nicastrin, Aph-1, and Pen-2 (Chyung et al., 2005). S3 cleavage finally results in the release of the Notch intracellular domain (NICD) into the cytoplasm from the surface. The NICD fragment translocates to the nucleus, where its interaction with a member of the CBF1/Su(H)/Lag1 (CSL) family of DNA-binding transcription factors and with the coactivator Mastermind allows it to function as a part of a transcriptional activator complex of target genes with a regulatory sequence recognized by the CSL protein

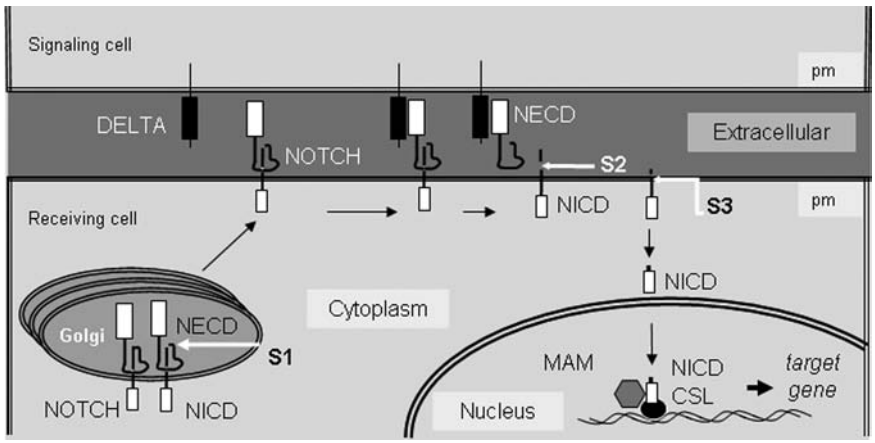

FIGURE 15.2 **Basic steps of the Notch signaling pathway.** Notch undergoes a Furin-dependent S1 cleavage in the Golgi to generate the mature receptor, a heterodimer of the Notch extracellular domain that is attached noncovalently to the remaining membrane-bound receptor. Interaction with a Delta Serrate Lag-2 ligand facilitates the separation of the Notch extracellular domain fragment and subsequent S2 and S3 cleavage. This releases the Notch intracellular domain, which translocates to the nucleus to displace a corepressor complex and to drive target gene expression with the DNA-binding CBF1/Su(H)/Lag1 protein and the coactivator Mastermind.

(see Figure 15.2). In the absence of the NICD fragment, CSL proteins have a critical role in maintaining the basal repression of their target proteins as part of corepressor complexes.

B. Endocytosis and Recycling Regulate Signaling by Delta Serrate Lag-2 Ligands

Experiments with the temperature-sensitive dynamin mutant *shibire* in Drosophila first suggested that the endocytosis of Delta and Notch in both the signal-sending and signal-receiving cell is essential for effective Notch signaling (Seugnet et al., 1997). Genetic analysis in Drosophila, zebrafish, Xenopus, and mouse has now defined a critical role for two families of RING (really interesting new gene) E3 ligases related to Neuralized (Deblandre et al., 2001; Lai et al., 2001; Pavlopoulos et al., 2001) and *mind bomb* (Itoh et al., 2003; Chen et al., 2004; Barsi et al., 2005; Koo et al., 2005a; 2005b; Lai et al., 2005; Le Borgne et al., 2005; Pitsouli et al., 2005; Wang et al., 2005), respectively, in ubiquitylating the intracellular domain of DSL ligands and promoting the endocytosis of the ligands (see Figure 15.2). Mosaic analysis has shown that this is an essential step in the effective activation of Notch in neighboring cells. The covalent conjugation of the 76 amino acid peptide ubiquitin to lysines in the intracellular domain of a membrane protein serves as a signal for recruiting endocytic proteins, like Epsin, that contain ubiquitin interaction motifs. Epsin is expected to provide a link to the endocytosis via a clathrin-mediated pathway (Hawryluk et al., 2006). Some tentative models have been suggested for the understanding of how the endocytosis of Delta-like and of Serrate/Jagged- and Lag2-related DSL family members might contribute to Notch activation (Chitnis, 2006).

One attractive model is based on the key role that the removal of the Notch ECD has in permitting subsequent S2 and S3 cleavages and in the release of the Notch ICD into the cytoplasm (Figure 15.3, A). Calcium chelators that disrupt calcium-dependent binding and cause disassociation of the NECD domain can artificially induce Notch activation. In addition,

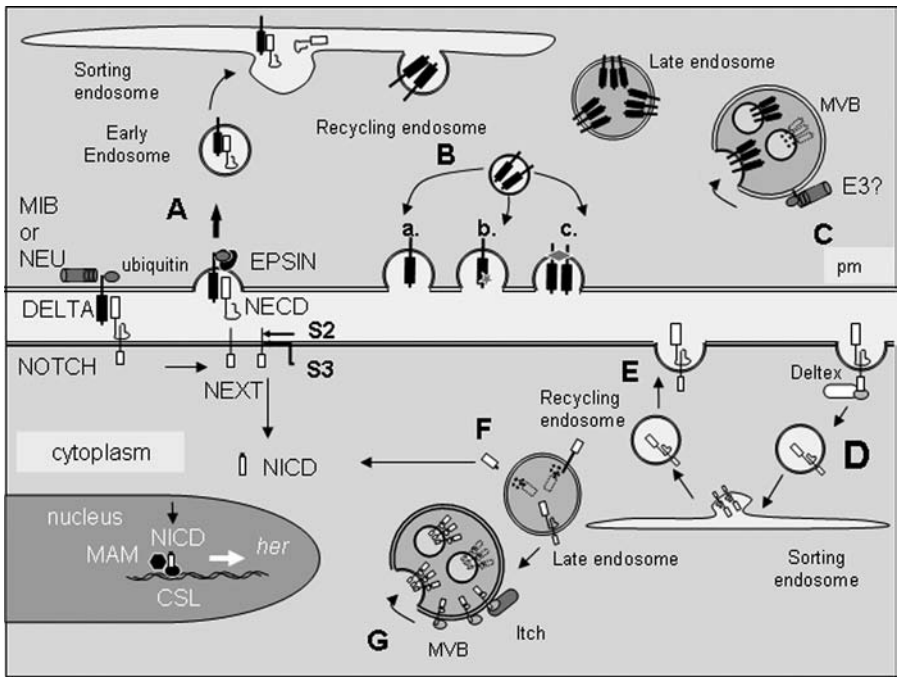

FIGURE 15.3 **The cell biology of Notch signaling. A,** Mind bomb *(Mib)*- or Neuralized *(Neur)*-mediated ubiquitylation and the endocytosis of Delta Serrate Lag-2 ligands like Delta, in association with epsin, facilitates the separation of the Notch extracellular domain and S2 and S3 cleavage. **B,** After the internalization of the early endosome and passage through a sorting endosome, some Delta is recycled. This may help Delta function by providing fresh Delta at the surface, a modified (star) Delta, or clustered Delta that interacts better with Notch. **C,** Delta that is not recycled may be sorted to the late endosome, where it is internalized in vesicles to form multivesicular bodies (MVBs) and ultimately destroyed in the lysosome. **D,** Notch, too, is ubiquitylated and internalized. **E,** After Notch enters the sorting endosome, it could return to the surface via a recycling endosome. **F,** Alternatively, Notch could go on to the late endosome. Notch extracellular domain degradation in the late endosome could trigger the release of the Notch intracellular domain fragment and the ligand-independent activation of Notch. G, Such activation would be prevented by the internalization of Notch into vesicles in the MVB. The internalization of cargo from the limiting membrane of the late endosome requires another ubiquitylation step. The Nedd4 family E3 ligase Itch facilitates the internalization of Notch in the MVB and eventual degradation in the lysosome. (See color insert.)

mutations that encode mutant forms of Notch that lack the NECD domain result in constitutively active forms of Notch. Finally, in Drosophila, signal-sending cells accumulate both Delta and the extracellular fragment of Notch in contexts in which they successfully activate Notch in a neighboring cell. On the basis of these observations and others that show that Epsin is essential for the delivery of the Delta signal, it is possible that, after strong binding of the DSL ligand with the NECD, Epsin-dependent endocytosis of the bound DSL ligand provides the mechanical force for the dissociation of the NECD domain, thereby facilitating subsequent S2 and S3 cleavage in the remaining Notch receptor. A variation of this model suggests that the initiation of the endocytosis of the bound DSL ligand–NECD complex may induce conformational changes that facilitate access of ADAM proteases to the S2 cleavage site.

The replacement of the Delta intracellular domain with the low-density lipoprotein receptor intracellular domain results in a form of Delta that can

deliver a signal that is independent of ubiquitylation and Epsin-mediated endocytosis (Wang et al., 2004). Because the low-density lipoprotein receptor has intracellular motifs that facilitate both endocytosis and recycling, this observation has been interpreted to suggest that endocytosis is important, because it eventually facilitates recycling. Because the loss of Epsin function does not affect the bulk endocytosis of Delta, it has been suggested that endocytosis coupled with recycling is required to return a more effective ligand to the surface. In this context, recycling may help provide a fresh supply of DSL ligand to the appropriate cell surface, where it can interact with Notch on a neighboring cell; it may return Delta that is dissociated from bound NECD, or, during the process of recycling, Delta may be modified in additional ways that make interactions between it and Notch on the neighboring cell more effective (see Figure 15.3, B). Independent of whether Delta endocytosis contributes mechanically by facilitating the removal of the NECD and/or indirectly through subsequent recycling, it is clear (as discussed later) that, eventually, the regulation of both endocytosis and of the recycling of DSL ligands contributes to the effective delivery of a signal to neighboring cells and that factors that regulate these trafficking events can influence the outcome of Notch signaling.

C. Endocytosis Regulates Notch Receptor Function

The function of the Notch receptor is also regulated by endocytosis. However, as with receptor endocytosis in many other signaling systems, the ubiquitylation and endocytosis of Notch is often associated with the degradation and downregulation of the signaling pathway (see Figure 15.3, D; Le Borgne, 2006). For example, Numb is a conserved membrane-associated protein that acts upstream of γ-secretase to block Notch signaling. It interacts with both Notch and α-adaptin, which is a subunit of the clathrin adaptor complex, to promote Notch endocytosis (Berdnik et al., 2002). In addition (or alternatively), Numb interacts with and prevents the plasma membrane localization of Sanpodo, a four-pass membrane protein; the localization of this protein on the plasma membrane is essential for Notch function (Hutterer et al., 2005; Langevin et al., 2005; Roegiers et al., 2005). Members of the Nedd4 family of E3 ligases, including Suppressor of Deltex (Su(dx)) in Drosophila and Itch in vertebrates, determine Notch degradation (Wilkin et al., 2004). In cell culture, Numb interacts with Itch, which is an E3 ligase that can ubiquitylate Notch and promote its endocytosis and degradation (McGill et al., 2003).

It should be noted that the presence of either a conserved intracellular downregulation targeting signal domain or a C-terminal PPXY motif in the Notch family receptors determines two alternate mechanisms by which the E3 ligases regulate Notch function (Shaye et al., 2005). The PPXY motif in Drosophila Notch determines internalization from the plasma membrane by Su(dx). By contrast, the downregulation targeting signal (DTS) domain of Lin12 contains an endocytic dileucine motif, and it mediates interactions with the *Caenorhabditis elegans* Su(dx) ortholog WWP-1. The ubiquitylation of flanking lysine by WWP-1 determines the internalization of this Notch family member from the limiting membrane of multivesicular bodies into intraluminal vesicles, where it is eventually degraded. It has been suggested that, if Notch is not internalized from the late endosome-limiting membrane, the NECD domain may be cleaved off in the acidified late endosome, thereby

resulting in the ligand-independent release of the NICD fragment into the cytoplasm (see Figure 15.3, F and G; Shaye et al., 2005). As indicated by the original experiments with the temperature-sensitive dynamin mutant *shibire* in Drosophila, Notch endocytosis also has a role in Notch activation. In this context, another RING E3 ligase, Deltex, has a positive role in Notch signaling. It functions downstream of the ligand and upstream of Su(H); however, the manner in which it promotes Notch activation remains unclear (Matsuno et al., 1995). It remains to be determined in what ways Notch endocytosis actually promotes activation.

D. Interaction in cis Interferes With Delta Serrate Lag-2 Ligand and Notch Function

Although the previous section has emphasized interactions of DSL ligands with Notch family receptors in trans (i.e., while on the surface of adjacent cells), these transmembrane proteins are also capable of interacting in cis (i.e., within membranes of the same cell; Micchelli et al., 1997). Interactions in cis, however, appear to result in nonproductive interactions. As a consequence, the expression of high levels of Delta in a cell simultaneously allows it to activate Notch in a neighboring cell while interfering with Notch's ability to be activated by Delta in a neighboring cell. A form of Delta that lacks its intracellular domain, DeltaΔICD, cannot interact with E3 ligases that promote its internalization and allow it to be an effective activator of Notch in neighboring cells. However, DeltaΔICD can still interact with Notch in cis, and this makes it an effective inhibitor of Notch receptor function in the cell in which it is expressed (Itoh et al., 2003).

As with the inhibitory effects of Delta on Notch function in cis, the expression of the Notch family receptor Lin-12 in *C. elegans* can interfere in cis with the function of DSL ligands, thereby preventing them from activating Lin-12 in neighboring cells. In cells that express both a DSL ligand and the Lin-12 receptor, mechanisms that internalize and degrade Lin-12 protein play a critical role in allowing ligands to effectively activate the Lin-12 in the neighboring cell (Shaye et al., 2005).

I. DETERMINING THE PRECISE FATE OF SINGLE CELLS

Although Notch signaling primarily regulates fate in adjacent cells, additional signaling mechanisms with a broader range of operation influence the outcome of Notch signaling. This section uses a series of examples to illustrate how, by working together with other signaling systems that can bias the outcome, Notch signaling can facilitate the specification of unique fates to cells with single-cell precision in a distribution that could not have been reliably achieved by either Notch signaling or the biasing mechanism alone.

A. Selection of a Central Cell Within A Proneural Cluster

The Drosophila epidermis develops more than 1000 bristles and other types of sensory organs during development. Some of these bristles, called *macrochaetes,* are conspicuously large, and they are located in stereotyped positions on the head and notum. Each macrochaete is derived from a single cell called a sense organ mother cell (SMC) as discussed for sensory organ precursors (SOPs) in the next section (Figure 15.4). In the wing imaginal disc, each SMC is, on

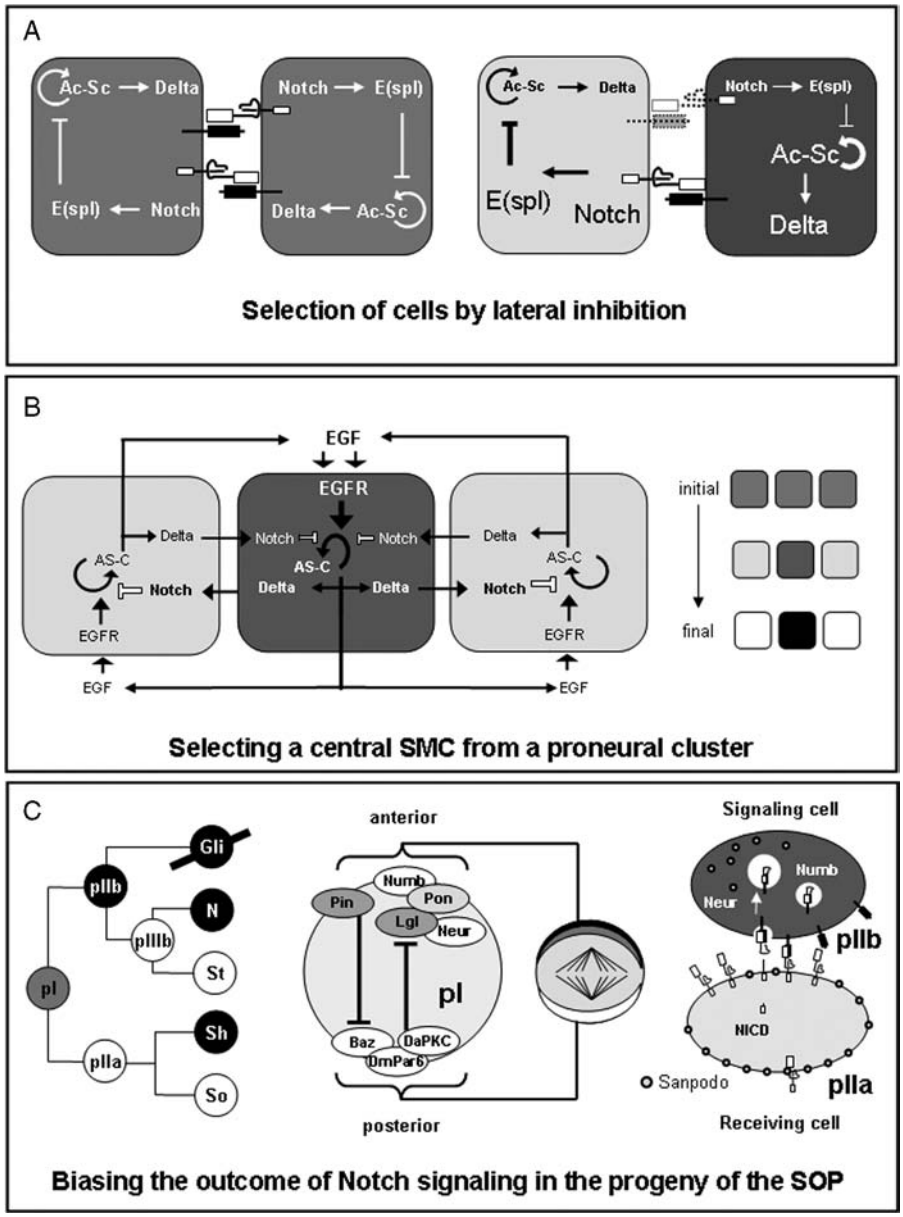

FIGURE 15.4 **Biasing the outcome of Notch signaling during lateral inhibition. A,** The basic negative feedback loop that can amplify small differences in potential between cells. After several reiterations of the loop, one cell becomes progressively better at signaling and the other better at receiving Notch activation. Eventually, the cell with lower Notch activity and higher *achaete-scute* function acquires a distinct fate from its neighbor with high Notch activation and low *achaete-scute* expression. **B,** Epidermal growth factor receptor signaling can facilitate autoregulation in the *achaete-scute* complex. *Achaete-scute* function could facilitate the development of a central bias by promoting the secretion of an epidermal growth factor receptor ligand. In the absence of such a bias, a cell at the edge of a cluster would be selected by lateral inhibition. **C,** The polarized segregation and inheritance of factors that can influence the trafficking and function of Delta and Notch can determine which cell is likely to become a dominant signaling and receiving cell. (Note: Additional factors that are not shown also contribute to the asymmetry in Delta–Notch function. Fates determined by relatively high Notch activation are shown as *white squares* in part B and *white circles* in part C.)

average, selected from a cluster of 20 to 30 cells called a *proneural cluster.* All of the cells in the proneural cluster initially acquire the potential to become SMCs by their expression of proneural genes like *achaete* and *scute,* which are part of the achaete–scute complex. These genes encode basic helix–loop–helix transcription factors, the expression of which promotes SMC fate. However, competitive interactions mediated by Delta–Notch interactions eventually restrict high levels of proneural expression and SMC fate to a single cell or sometimes two cells within the proneural cluster. High levels of proneural expression are first restricted to a smaller subset of cells within the proneural cluster, called a *proneural field;* then, within this field, the cell that expresses the highest level of *achaete scute* expression begins expressing *asense* (another proneural gene in the achaete–scute complex) and acquires an SMC fate (Culi et al., 2001).

The initial expression of proneural genes in stereotypical clusters is determined by the combinatorial function of heterogeneous "prepattern" genes; however, the sustained high expression of proneural genes in a subset of cells depends on self-stimulation or autoregulation by the proneural genes. However, not all cells in the proneural cluster can successfully autoregulate, because although the proneural genes promote their own expression, they also drive the expression of the Notch ligand Delta. The expression of Delta activates Notch in neighboring cells, and the release of the transcriptionally active NICD fragment in these cells drives the expression of genes in the Enhancer of split (E(spl)) complex. These genes encode another class of basic helix–loop factors that function together with corepressors like Groucho to inhibit proneural function. As a consequence, each cell that expresses proneural factors simultaneously tries to increase its own level of proneural expression by autoregulation, whereas Delta expression activates Notch, and the expression of E(spl) inhibits proneural function in the neighboring cells. This process, in which each cell in the cluster tries to inhibit its neighbor from adopting the same fate, is referred to as *lateral inhibition* (see Figure 15.4, A). Cells in the proneural cluster compete to drive proneural expression, and, eventually, cells with the least Notch activation and the strongest proneural autoregulation acquire high enough levels of proneural activity to be selected as an SMC. When Delta–Notch signaling fails in this context, too many cells in the proneural cluster are permitted to adopt an SMC, and this can result in the formation of too many macrochaetes. Furthermore, if, early in the process, a prospective SMC is eliminated, a neighboring cell in the proneural cluster that would otherwise not have won the competition to become an SMC would now be released from inhibition and be able to adopt this fate instead. This scheme, by which a single cell is selected for an SMC fate in a "proneural cluster" by lateral inhibition, is paradigmatic of the role played by Notch signaling in many tissues many times during development, and it represents an evolutionarily conserved strategy for defining the number of differentiating cells within a field in which earlier "prepatterning" mechanisms have defined the boundaries of a domain in which cells acquire the potential to adopt a particular fate. A very similar strategy defines how, for example, neuroblasts are selected in the developing Drosophila central nervous system or how early neurons are specified in the zebrafish and Xenopus neural plate (Chitnis et al., 1995; Lewis, 1996).

What determines which cell is eventually selected in a cluster and how it is reliably selected in a particular location within a large proneural cluster? It is

important to note that, in the absence of additional biasing mechanisms, a central cell would be least likely to be selected by lateral inhibition, because cells capable of inhibiting it surround it. By contrast, cells at the edge of a competent cluster have neighbors that do not deliver inhibitory signals, and they are more likely to be selected by lateral inhibition acting on its own. One strategy that could provide a central bias is the secretion of a diffusible factor that promotes proneural function within a proneural cluster. If, in addition to promoting lateral inhibition by directing the expression of the Notch ligands (like Delta), the proneural factors also directed the expression of a diffusible factor that promotes proneural autoregulation in neighboring cells, central cells in a cluster would be most exposed to the diffusible factor, and they would be most effective at autoregulation (see Figure 15.4, *B*). In this context, lateral inhibition would amplify small differences in autoregulatory potential within a field of competent cells, and this could eventually facilitate the selection of a central cell. This simple strategy for generating a central bias appears to be employed in the specification of the SMCs selected within a central proneural field, where the secretion of diffusible EGF receptor (EGFR) ligands driven by proneural genes can bias the efficacy of proneural autoregulation (Culi et al., 2001). The analysis of enhancer elements in the *achaete–scute* complex identified distinct enhancers responsible for coordinating inputs from heterogenous factors that help initiate proneural gene expression within cell clusters at stereotyped locations. In addition, a distinct SMC enhancer was identified that is responsible for the particularly high expression of proneural genes in the proneural field. Expression directed by this enhancer is dependent on proneural autoregulation; however, expression directed by the autoregulatory SMC enhancer is, in addition, very sensitive to the level of EGFR signaling through the Ras/Raf/mitogen-activated protein kinase pathway. Limitations in the sensitivity of tools available to detect EGFR activation make it is difficult to visualize patterns of EGFR activation within the proneural clusters, where low levels of activity appear to be sufficient to promote the efficacy of proneural autoregulation. However, the manipulation of EGFR signaling clearly demonstrates that exaggerated EGFR signaling can enhance expression directed by the SMC enhancer and that the broad expression of Argos, a diffusible EGFR antagonist, can prevent the specification of macrochaetes.

B. Biasing Binary Cell-Fate Decisions in the Sensory Organ Precursor Lineage

SOPs or the pI cells on the dorsal surface of the fly thorax (notum) undergo a series of stereotyped asymmetric divisions to generate cells that make cells of adult mechanosensory organs (see Figure 15.4, C). The first of the asymmetric divisions is along the mediolateral axis, and this generates an anterior pIIb cell and a posterior pIIa cell. The pIIa cell divides once more to form a socket and a shaft cell, which are the external cells of the mechanosensory organ. The pIIb cell divides twice to produce the internal cells of the sensory organ: the first division produces a glial cell and the pIIIb cell, and then the pIIIb cell divides to form a sensory neuron and a sheath cell. The distinct fate of the cells after each division of the SOP and its progeny is determined by the relative amount of Notch activation in the siblings. For example, pIIa fate is dependent on high Notch activation, whereas pIIb fate is dependent on low Notch activation. Each sibling expresses both Delta and Notch, and each could, in principle, compete to acquire a dominant role in either delivering or receiving a Notch

signal. However, the asymmetric distribution of Numb, Neuralized, and Sanpodo, which is determined by planar polarity signaling mechanisms, ensures that the anterior pIIb cell becomes more effective at delivering the Delta signal, and that the posterior pIIa cell becomes more effective at having its Notch activated (see Figure 15.4, C; Langevin et al., 2005; Roegiers et al., 2005).

How is this achieved? During prometaphase, planar polarity mechanisms segregate the Pin protein to the anterior cortex of the pI cell; this is opposite of the components of Par complex (Bazooka [D-Par3], DaPKC, and DmPar6), which localize on the posterior cortex. Pins restricts the localization of Baz to the posterior cortex of the dividing pI cell, and the segregation of Baz along with Da PKC and DmPar6 at the posterior cortex restricts another factor, Lethal giant larvae (Lgl), to the opposite anterior cortex. Lgl in turn recruits Partner of Numb (Pon) and Neuralized to the anterior cortex. As a consequence, after mitosis, Numb and Neuralized are segregated to the anterior pIIb cell. Numb prevents the membrane localization of Sanpodo and inhibits function of Notch in the pIIb cell, as discussed previously. In addition, the segregation of Neuralized to the pIIb cell allows this cell to internalize Delta and to become effective at signaling with Delta to its neighbor. By contrast, the absence of Numb and of the plasma membrane localization of Sanpodo makes Notch effective in the pIIa cell, whereas the absence of Neuralized prevents this cell from delivering an effective Delta signal to its sibling. In this manner, planar polarity mechanisms ensure that, for the pII progeny of all the SOPs on the developing notum, the anterior sibling acquires a pIIb fate, whereas the posterior sibling acquires a pIIa fate (Langevin et al., 2005; Roegiers et al., 2005). The coordinated fate of individual SOP progeny eventually ensures the coordinated orientation of all of the sensory bristles in the notum.

In addition to asymmetries in Delta and Notch endocytosis mediated by Numb, Neuralized, and Sanpodo, another mechanism acting in parallel establishes an asymmetry in Delta recycling in the pIIa and pIIa cells (Emery et al., 2005). Shortly after the division of the SOP, a Rab11-dependent recycling center is established around the centrosome in the pIIb cell. The establishment of this center is dependent on the accumulation of the Rab11-binding protein Nuclear fallout (Nuf; the Drosophila homolog of vertebrate Arfophilin) on the centrosome. The association of Nuf with the centrosome is inhibited in the pIIa cell. As a consequence, soon after division, the pIIb cell begins recycling Delta that was internalized before or during mitosis, thus quickly establishing itself as the dominant signaling cell. Additional studies have demonstrated that Sec15, which is a component of exocyst complex and which ensures that recycled proteins are delivered back to the plasma membrane, is essential for effective Delta signaling (Jafar-Nejad et al., 2005). These studies not only define the asymmetric establishment of a recycling center as another mechanism for ensuring that the anterior SOP daughter cell is established as the dominant signaling cell, but they also underscore the critical role of Delta recycling in determining the efficacy with which it is able to activate Notch in a neighboring cell.

C. Specification of 1°, 2°, or 3° Fates in Vulval Precursor Cells

Cell-fate specification in vulval precursor cells (VPCs) illustrates another interesting example of the way that the Notch family receptor Lin-12 and EGFR signaling work together to specify cell fate (Figure 15.5; Sternberg, 2004). In particular, it illustrates how the regulation of Lin-12 trafficking by EGFR helps

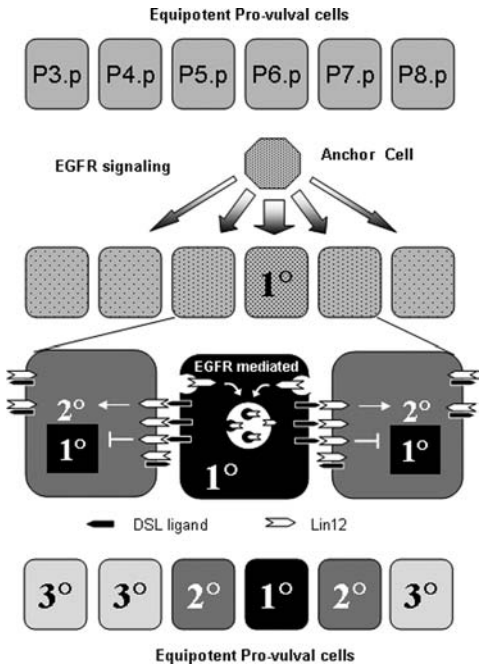

FIGURE 15.5 **The specification of distinct fates in vulval precursor cells by a combination of epidermal growth factor receptor (EGFR) and Lin-12 signaling.** EGFR signaling in the P6.p precursor closest to the source of inductive signals induces 1° fate and facilitates the internalization and degradation of Lin-12. This facilitates the delivery of a signal to Lin-12 in neighboring P5.p and P7.p cells, where the activation of Lin-12 target genes promotes 2° fate and inhibits 1° fate. In the absence of either effective EGFR or Lin-12 signaling, the cells acquire 3° fate.

to reliably specify a precise pattern of VPC fates in *C. elegans*. In the third larval stage of *C. elegans,* six contiguous epidermal cells (P3.p, P4.p, P5.p, P6.p, P7.p, and P8.p) become competent as VPCs to adopt one of three cell fates: 1°, 2°, or 3°. The 1°, 2° cells look similar but undergo distinct patterns of division to contribute to the formation of the vulva. The 3° cells fuse with the hypodermis and become a part of the epidermal syncytium. Strong activation of the EGFR induces a 1° fate and prevents a 2° in the VPCs, whereas the activation of the Notch-related Lin-12 receptor promotes a 2° fate and inhibits 1° fate. Both EGFR and Lin-12 signaling inhibit 3° fate. As a consequence of antagonistic signaling and some interesting cross regulation, the P3.p, P4.p, P5.p, P6.p, P7.p, and P8.p cells are reliably specified, respectively, with 3°, 3°, 2°, 1°, 2°, and 3° fates.

The P6.p cell is closest to the anchor cell, which is a somatic gonad cell that is the source of diffusible EGF signals. Proximity to the source of EGF signals most effectively activates EGFR in the P6.p cell, although it is a little less effectively activated in neighboring P5.p and P7.p cells. The strong activation of EGFR promotes 1° fate in the P6.p cell and at the same time facilitates the activation of Lin-12 in the neighboring P5.p and P7.p cells. The activation of Lin-12 promotes 2° fate in the P5.p and P7.p cells, whereas the absence of both effective EGFR and Lin-12 activation in the remaining P3.p, P4.p, and P8.p cells allows these cells to adopt a 3° fate.

EGFR signaling has an unusual role in ensuring that Lin-12 activation is limited to the P5.p and P7.p cells. In addition to promoting 1° fate, EGFR

signaling promotes the internalization and degradation of Lin-12 (Shaye et al., 2002). The expression of Lin-12 in a VPC interferes with that cell's ability to deliver an effective lateral signal to the Lin-12 expressed in a neighboring cell. Although all VPCs express Lin-12 and make the protein, the EGFR-dependent internalization and degradation of Lin-12 in the P6.p cell ensures that this cell specifically can most effectively activate Lin-12 in the neighboring P5.p and P7.p cells.

II. PATTERNING CELL FATE AT COMPARTMENT BOUNDARIES

So far, examples of patterning events have been discussed to illustrate how Notch signaling interacts with other mechanisms to reliably specify the fate of single cells in characteristic patterns within a tissue. In the next section, patterning events will be discussed in which DSL ligands expressed in one tissue compartment activate Notch in an adjacent tissue compartment to induce a unique cell fate in a single row of cells at the boundary of the two compartments. In some contexts, the activation of cells on one side of a boundary results in the reciprocal activation of adjacent cells on the other side of the boundary. In this context, the boundary cells can become signaling centers for organizing cell fate and proliferation in the adjacent tissue. Furthermore, in addition to regulating fate through signaling mechanisms, interactions between DSL ligands and Notch may also have a critical role in organizing cytoskeletal elements at the boundary to regulate cell morphology and to create a physical barrier to the movement of cells from one tissue compartment into another.

A. sim Expression at the Mesoderm–Ectoderm Boundary

During early dorsal–ventral (DV) patterning in Drosophila, Notch signaling helps to define mesectodermal fate in a single row of cells at the mesoderm–ectoderm boundary. Maternal patterning mechanisms establish a DV gradient of activity for the transcription factor Dorsal by regulating its nuclear translocation (Figure 15.6; Stathopoulos et al., 2002). Ventrally, where it is primarily nuclear, Dorsal effectively drives the expression of the transcription factor Twist. Dorsal synergizes with Twist to drive *snail* expression in a sharply defined ventral territory, where Snail, functioning as a repressor, has a critical role in specifying mesoderm fate by inhibiting the expression of genes that define neuroectoderm or mesectoderm fate. In the lateral blastoderm and thus in the absence of *snail* repression, intermediate levels of nuclear Dorsal and Twist contribute to the expression of genes that define neuroectoderm and mesectoderm fate. However, in addition to Dorsal and Twist, Notch activation is required to drive the early expression of the mesectoderm gene *sim* in the lateral blastoderm (Martin-Bermudo et al., 1995). In the absence of Notch activation, the DNA binding factor Su(H) associates with corepressors to represses *sim* expression, and *sim* expression is only permitted in cells in which Notch activation allows NICD to overcome basal repression (Morel et al., 2000).

In this context, the precise activation of *sim* in a single stripe of ectodermal cells at the mesoderm–ectoderm boundary is dependent on Notch activation by the Delta expressed in the adjacent mesoderm. Although Delta is broadly expressed in the embryo, it is only capable of activating *sim* expression at the mesoderm–ectoderm boundary cells because of sharp differences in

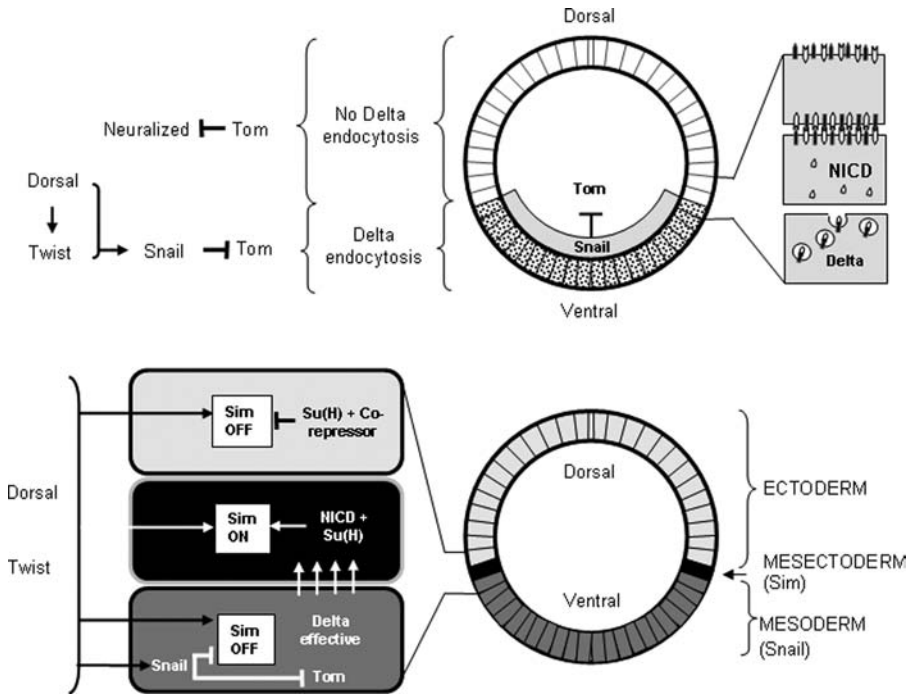

FIGURE 15.6 The determination of single minded *(sim)* expression at the ectoderm–mesoderm boundary. Dorsal and Twist determine the expression of the repressor *snail* in a sharply defined ventral region that defines the prospective mesoderm. *snail* inhibits *tom* expression and permits the Neuralized-mediated endocytosis of Delta in the mesoderm *(speckled)*. *tom* is expressed in the ectoderm, where Neuralized function is inhibited, and Delta remains on the cell surface. The effective activation of Notch in the ectoderm boundary cells by Delta in the adjacent mesoderm relieves Su(H)–mediated repression and activates sim in the boundary cells.

the ability of mesoderm and ectoderm cells to internalize Delta and to deliver an effective signal to neighboring cells (Bardin et al., 2006; De Renzis et al., 2006). Maternal Delta is prominently expressed on the cell surface in the preblastoderm embryo; however, cellularization and the initiation of zygotic gene expression are accompanied by a sharp difference in Delta distribution in the ectoderm and adjacent mesoderm: although Delta redistributes to endocytic vesicles in the mesoderm, it remains on the cell surface in the ectoderm. The internalization of Delta is dependent on the E3 ligase Neuralized, and it permits Delta in the mesoderm to effectively activate Notch in the neighboring cells. However, this sharp difference in the ability of ectoderm and mesoderm cells to internalize Delta is not dependent on the restricted expression of Neuralized in the ventral mesoderm compartment, because the distribution of *neuralized* transcripts extends at low levels dorsally into the ectoderm. Rather, the sharp difference in the ability to internalize Delta and to deliver an effective signal is related to the differential expression of *Bearded (Brd)* family genes like *tom,* which encode inhibitors of Neuralized function. Dorsally, *tom* is expressed in the ectoderm, where it prevents Neuralized function and accounts for the predominant cell-surface expression of Delta. Ventrally, in the mesoderm, however, *snail* represses *tom* expression and permits Neuralized function. Neuralized-mediated Delta endocytosis permits mesoderm cells to activate Notch in adjacent cells, where NICD acts together with Dorsal and

Twist to drive *sim* expression. However, because *snail* also represses *sim* in the mesoderm, *sim* expression gets effectively restricted to a single row of cells at the mesoderm–ectoderm boundary, where Notch is activated by Delta that is expressed in the cells of the adjacent mesoderm. *tom* prevents the access of Neuralized to Delta rather than interfering with its catalytic function, and the forced expression of *tom* not only inhibits wild-type Neuralized, but it also suppresses a phenotype that is induced by a dominant-negative Neuralized. No functional homologues of *tom* have yet been identified in vertebrates.

The regulation of Neuralized function by *Brd* genes also facilitates the selection of the SOP cell within a proneural cluster. Notch activation in prospective non-SOP cells drives the expression of *Brd* genes, thereby inactivating Neuralized and making these cells less effective at delivering Delta-mediated lateral inhibition to their neighbors. By contrast, in the absence of Notch activation, Su(H) maintains the repression of *Brd* genes in the prospective SOP cell, thus facilitating Neuralized function and making it more effective at delivering lateral inhibition to neighboring cells (Bardin et al., 2006).

B. Specification of Dorsal–Ventral Boundary Cells in the Wing Margin

The role of Notch signaling at the DV compartment boundary in the wing imaginal disc is paradigmatic of the function of Notch signaling in boundary formation (Figure 15.7). The dorsal compartment of the wing imaginal disc is characterized by the expression of the transcription factor Apterous (Diaz-Benjumea et al., 1993). It determines the expression of the DSL ligand Serrate and the glycosyltransferase Fringe in the dorsal compartment. Fringe modifies the extracellular domain of Notch-inhibiting Serrate–Notch interactions and facilitates Delta–Notch interactions (Panin et al., 1997). As a consequence of this modification, Serrate cannot activate Notch in surrounding dorsal cells; however, the absence of Fringe in the ventral cells allows Serrate to effectively activate Notch in adjacent ventral cells at the DV compartment boundary. The activation of Notch drives the expression of Delta in the ventral boundary cells. These Delta-expressing cells provide the reciprocal activation of dorsal cells at the compartment boundary, where the modification of Notch by Fringe facilitates interaction with this DSL ligand. The activation of Notch in dorsal cells promotes more expression of Serrate in these cells, thereby allowing them to even more effectively activate Notch and drive Delta expression in adjacent ventral cells. The resulting autoregulatory loop sustains high levels of Notch activation and DSL ligand expression in cells on both sides of the DV boundary (see Figure 15.7, A). The sustained Notch activation activates the expression of additional target genes, including *wingless,* at the DV compartment boundary. Wingless (Wg) is secreted from the DV boundary, and it signals adjacent cells both dorsally and ventrally, where it regulates cell proliferation. In addition, it induces the expression of proneural genes in adjacent domains to generate proneural clusters in which sensory bristles are eventually established on either side of the wing margin. In the absence of the interactions that establish a signaling center at the DV boundary, the wing imaginal disc cannot grow, and sensory bristles are lost in sections of the margin where *wingless* expression is absent.

The high levels of Notch activation established by reciprocal Serrate–Notch and Delta–Notch interactions drive *wingless* expression at the DV

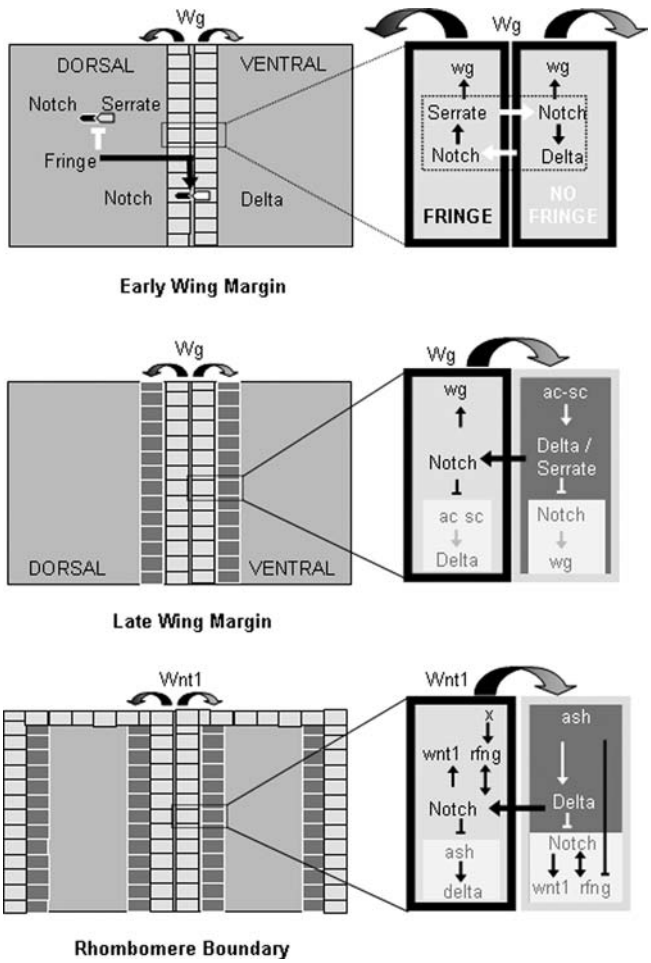

FIGURE 15.7 Similarities in the mechanism of Notch signaling at the Drosophila wing margin and in the zebrafish rhombomere boundaries. Fringe inhibits effective interaction between Serrate and Notch, but it facilitates interaction with Delta. During the early development of the wing margin, Serrate does not activate Notch within the dorsal compartment, where Notch is expressed together with Fringe. However, effective interactions are possible at the boundary, with Notch in ventral cells that do not express Fringe. The activation of Notch drives Delta expression in ventral boundary cells. Delta activates Notch in dorsal boundary cells, thereby driving Serrate expression and setting up a positive feedback loop. Eventually, Notch activation also drives *wingless* expression in boundary cells. During the late development of the wing margin, *wingless* diffuses laterally to drive *achaete-scute* expression in cells that are adjacent to the boundary. *Achaete-scute* factors drive Delta/Serrate expression in paraboundary cells, where these Delta Serrate Lag-2 ligands have a critical role in maintaining Notch activation and the secretion of *wingless* from boundary cells. Meanwhile, they also have a critical role in the cis inhibition of Notch and in *wingless* expression in paraboundary cells. A similar set of interactions maintains Notch activation and Wnt expression in boundary cells, and the cis inhibition of Notch prevents the spread of boundary fate to neighboring cells.

boundary. The Wg signals, in turn, facilitate a second feedback loop that both positively maintains their own expression at the boundary and at the same time has a critical role in preventing the spread of Notch activation and the Wg signaling center to adjacent cells (see Figure 15.7, B; Micchelli et al., 1997). As discussed previously, Wg induces proneural gene expression in cells

that are adjacent to the boundary, and they in turn drive Delta and Serrate expression in these cells. In this context, Delta and Serrate have critical roles in the cis inhibition of Notch signaling (Micchelli et al., 1997). As long as at least one Notch ligand is expressed, Notch is activated in adjacent cells. However, a loss of Delta can interfere with its critical cis inhibitory role, thereby allowing the progressive spread of Notch activation and the source of Wg into adjacent cells. In addition to the cell-autonomous role for Delta and Serrate in inhibiting Notch activation in cells that are adjacent to boundary cells, Su(H) has a critical role in maintaining the basal repression of Notch target genes. Nubbin/POU domain transcription factor also contributes to the basal repression of Notch target genes (Neumann et al., 1998). In addition, Crumbs, which is a factor that is known primarily for its role in apical–basal polarity, prevents unregulated γ-secretase activity and ensures that Notch S3 cleavage is restricted to boundary cells (Herranz et al., 2006). Additional genes like *l(2)giant discs* also contribute to the repression of Notch activity, although the mechanism by which this occurs remains unclear (Klein, 2003). Together, these studies indicate that a number of factors are critical for restricting Notch activity and the Wg signaling center to boundary cells at the wing margin.

C. Boundaries and Morphogenesis

The interaction of Notch with its ligands in neighboring cells at the DV compartment boundary has a morphologic role in defining a smooth boundary that serves as a barrier to the mixing of cells between the dorsal and ventral compartments. The loss of Notch function results in an irregular boundary, and cells from one compartment can invade the opposite compartment. The bidirectional nature of Notch signaling at the DV boundary suggests that Notch activation is unlikely to promote cell sorting by promoting a dorsal- or ventral-specific cell affinity. Consistent with this expectation is the fact that the loss or gain of Notch function does not promote the selective segregation of cells in either the dorsal or ventral compartment.

As an alternative to a compartment-specific affinity model, it has been suggested that Notch activation may produce a boundary-specific affinity, with compartment-specific genes providing additional input to define a dorsal- or ventral-specific boundary fate. However, ectopic stripes of enhanced Delta–Notch interactions are sufficient to generate smooth boundaries entirely within a given compartment. This can be achieved, for example, by generating an ectopic clone of cells expressing Delta dorsally or by generating a stripe of Fringe expressing cells ventrally. Such observations have led to a "fence" model that does not rely on differential cell affinities to generate boundaries and that could operate autonomously within the dorsal or ventral compartment (Major et al., 2005).

The "fence" model suggests that Delta–Notch interactions organize cytoskeletal elements in boundary cells, thereby creating a barrier that prevents the movement of cells from one compartment to another. It postulates that the tension generated by cytoskeletal elements may ensure that boundaries are smooth. The analysis of the potential role of various cytoskeletal elements revealed that relatively thick bundles of F-actin can be visualized within a specific developmental window at the DV boundary. These F-actin bundles are specifically associated with Notch-dependent compartment boundaries. They

are not seen, for example, at the anterior–posterior compartment boundary in the wing imaginal disc, where compartment-specific cell affinities play a key role in the segregation of cells at the boundary. The F-actin fence can be visualized by its colocalization with a nonmuscle type II myosin and with Enabled, a tyrosine kinase that regulates actin polymerization. Although it is not clear whether these characteristic F-bundles are associated with dorsal or ventral cells at the boundary, it is clear that their polarized distribution at the boundary is associated with the face of the cells in which interactions of Notch with its ligands are maximized. The strongest evidence for the role of F-actin at the DV boundary comes from *capulet* mutant clones. *Capulet* (*capt;* also known as *act up*) consistently and specifically disrupts the DV compartment boundary under partial loss-of-function conditions. *Capt* is a Drosophila cyclase-associated protein (Baum et al., 2000; Benlali et al., 2000) that restricts apical actin polymerization in epithelial cells (Baum et al., 2001). The mechanism by which Notch interactions with its DSL ligands organize F-actin at the boundaries remains unclear. However, it appears that it may be not be related directly to the signaling function of Notch and that the ectopic expression of a constitutively active form of Notch is not as effective at generating an effective boundary. There is a conserved PSD95/Dlg/ZO-1 (PDZ) binding motif in the C-terminal of some of DSL ligands (Wright et al., 2004), and the identification of PDZ-interacting membrane-associated guanylate kinase-like homologues (MAGUK) proteins like Discs large (Dlg), which are stabilized by Delta at the adherens junctions and which inhibit the mobility of cells (Six et al., 2004), raises the possibility that interactions mediated by this C-terminal PDZ-binding motif and/or by additional intracellular domains may contribute in some way to the stabilization of cells at the boundaries. However, this remains an untested hypothesis for now.

D. A Role for Notch in Rhombomere Boundary Formation

In the vertebrate hindbrain, rhombencephalon early patterning mechanisms define discrete compartments called rhombomeres (Moens et al., 2002). The expression of rhombomere-specific genes shows that these tissue compartments are specified soon after gastrulation by 10 hours postfertilization in zebrafish. However, 8 hours later, by the 18-somite stage, the rhombomeres become apparent as bulges with discernible morphologic boundaries. The morphologic changes are paralleled by subtle changes in rhombomere-specific gene expression: the ragged, uneven edges of gene expression at the rhombomere boundaries are transformed to well-defined sharp edges. These changes are in part the result of the fact that cell movements are constrained by compartment boundaries. However, it should be noted that cells can switch off the rhombomere-specific expression of their original compartment and turn on the rhombomere-specific expression of a new compartment as they move from one compartment to another, thus masking some limited movement across boundaries (Cooke et al., 2002).

At least two mechanisms contribute to the segregation of cells in a rhombomere-specific manner and to the formation of morphologic boundaries between these rhombomeres. One mechanism is based on repulsive interactions mediated by the ephrin ligands and Eph receptors that are expressed, respectively, in even- and odd-numbered rhombomeres (Cooke et al., 2002). By 18 hours postfertilization, when morphologic boundaries start to become apparent, a

second Notch-dependent mechanism that is similar to the one described in the Drosophila wing imaginal disc appears to come into operation (see Figure 15.7). At this time, a zebrafish homolog of *fringe* called *radical fringe (rfng*) starts being expressed at the rhombomere boundaries (Amoyel et al., 2005). *rfng* is expressed along with Wnt homologues like *wnt1;* however, *wnt1* expression, especially at this stage, is broader and not as clearly restricted to boundaries. The expression of *rfng* is, for the most part, complementary to the zebrafish *acute scute* homologs *asha* and *ashb* and to the zebrafish atonal homolog *ngn1,* which, at this stage, are relatively broadly expressed in the rhombomeres. These proneural genes impart competence for neurogenesis in the rhombomeres.

By 24 hours postfertilization, the expression of *rfng* remains restricted to rhombomeres. However, in addition, *wnt1* expression will also become more restricted to boundaries than it was at 18 hours postfertilization. At the same time, the expression of the proneural gene *ashb* and the delta genes *deltaA* and *deltaD* become restricted to cells flanking the boundaries. This restriction process is dependent on *wnt1,* proneural, and Delta function, and it appears to operate like the restriction mechanism operating in the wing margin. In an analogous manner, it has been suggested that Delta activates Notch particularly well in boundary cells that have begun to express *rfng*. Notch activation, together with an as-yet-unidentified factor X, drives *rfng* expression in the boundary cells, thus maintaining it as a cell where Delta can effectively interact with Notch. Notch also drives *wnt1* expression in these cells, and it diffuses to neighboring cells, where it drives proneural gene expression. The proneural genes drive Delta gene expression in these cells, where the Notch ligands have a critical dual role in maintaining high levels of Notch activation in the adjacent boundary cells while autonomously inhibiting Notch activation in the cell's boundary, where they are expressed. In the absence of effective Wnt signaling, proneural function, or subsequent Delta function in cells that are adjacent to the rhombomere boundaries, the cis inhibitory mechanism that restricts Notch activation and boundary gene expression to rhombomere boundaries fails, and "boundary gene" expression extends to cells that are progressively further from rhombomere boundaries but that can still be recognized by the rhombomere-wide expression of rhombomere-specific genes like krox20. The failure to restrict boundary gene expression and to establish restricted signaling centers eventually affects the morphogenesis of rhombomere boundaries and late neurogenesis in the domains that flank the boundaries (Amoyel et al., 2005).

Notch signaling not only has a role in establishing Wnt signaling centers at rhombomere boundaries, it also has a role in the morphogenesis of rhombomere boundaries. Although its role is currently poorly defined, mosaic analysis shows that cells expressing constitutively active forms of Notch tend to segregate to boundary regions, whereas cells expressing constructs that are expected to autonomously interfere with Notch activation tend to segregate away from boundaries (Cheng et al., 2004). Future studies will likely determine whether Notch participates in boundary-forming mechanisms that establish actin fences, which has been recently suggested to take place in Drosophila.

E. Somitogenesis: Notch Signaling and Synchronized Oscillations

In the examples of Notch signaling that have been considered so far, the activation of Notch by a ligand expressed in a neighboring cell either inhibited ligand

expression or promoted ligand expression in the Notch-activated cell. In this context, negative feedback facilitated Notch's role in lateral inhibition, whereas positive feedback involving downstream target genes helps establish an autoregulatory loop and a self-sustaining signaling center at boundary regions. This section examines a temporal aspect of the feedback mechanism in which a delay in negative feedback on target gene expression establishes and synchronizes oscillations in Notch target gene expression (Giudicelli et al., 2004).

Cells in the vertebrate presomitic mesoderm are capable of expressing a subset of Hairy enhancer of split-related genes (HER genes in zebrafish; HES genes in most other vertebrates) in response to Notch activation by ligands that are expressed in neighboring cells and, additionally, at some basal level in the absence of cell–cell interactions. These HES factors function as repressors of their own transcription, and, after they accumulate to a critical level, they shut off their own transcription until factors that promote their transcriptional activation overcome the repression and/or the HES protein levels fall below the threshold required for effective repression. In this context, transcription and then the translation of freshly synthesized HES factors follow after a delay that allows for the substantial accumulation of HER transcript before the accumulation of the freshly translated HES repressor is finally able to shut off its own transcription again. Although the basal transcription of HES genes and the delay in feedback inhibition can autonomously sustain oscillations to some degree, HES gene expression is also driven by Notch activation as a result of interactions with ligands in neighboring cells. The interactions between cells play a critical role not only in sustaining the oscillations during expression but also in synchronizing oscillations in adjacent cells. The synchronization is a predictable outcome of oscillator coupling, which is when one oscillating system is able to influence the behavior of another similar oscillating system (Jiang et al., 2000).

The synchronized oscillations of Notch target gene expression in the presomitic mesoderm define the molecular basis of a predicted clock in the theoretical "clock and wave front" model, which was suggested many years ago as a mechanism for the progressive distinguishing of the periodic segments of mesoderm, called *somites,* from the unsegmented presomitic mesoderm (Cooke et al., 1976). This model suggested that, as cells in presomitic mesoderm undergo periodic oscillations, a wavefront of differentiation passes caudally, and somite boundaries are specified in presomitic cells that are in a defined phase of their oscillation. It now known that the oscillation of Notch target genes is sustained by a minimum threshold of fibroblast growth factor (FGF) signaling in the presomitic mesoderm. As rostral presomitic mesoderm cells become progressively separated from the source of FGF in more caudal domains, FGF signaling drops below a threshold that is required to sustain HES oscillations, and cells remain locked in the phase at which the wavefront of depleted FGF signaling finds them. Presomitic cells at a particular phase of their oscillation begin a sequence of events that eventually defines a somite boundary (Dubrulle et al., 2001).

III. CONCLUDING REMARKS

A detailed understanding of the role of Notch signaling in a wide range of tissue contexts, which is often deduced from the complex genetic analysis of model

systems like Drosophila and *C. elegans,* has historically provided a very useful framework for understanding the role of Notch signaling in new contexts, particularly in vertebrate model systems, in which more limited genetic analysis was possible. Many examples of Notch function from Drosophila and *C. elegans* were selected to illustrate the details of how Notch signaling operates in a wide range of contexts to determine cell fate at the single-cell level. These examples are likely to serve as useful models as we continue to explore the role of similar genetic regulatory networks in vertebrates, including humans.

For the past two decades, there has been an explosion in our recognition of the remarkable evolutionary conservation of patterning mechanisms and genetic regulatory networks. A particularly exciting challenge for developmental biologists is to carefully apply the lessons about Notch signaling from one developmental context to another, because superficial similarities may sometimes be misleading, or deeper examination may sometimes reveal similarities that were not immediately apparent. The discovery of vertebrate Notch is a good example in this regard. In Drosophila, Delta–Notch signaling is well known for its role in determining a neuroblast fate in the neuroectoderm, where lateral inhibition mediated by Delta inhibits cells in a proneural cluster from adopting a neural fate and forces them to adopt an epidermal fate instead. The Xenopus homolog of Notch was identified with the expectation that it might provide insight into mechanisms of neural induction, which allows ectodermal cells to adopt a neural fate instead of an epidermal fate (Coffman et al., 1993). However, the analysis of Xenopus Delta revealed that Notch had a more obvious role in the selection of cells that become neurons within the neuroectoderm (Chitnis et al., 1995). Now, vertebrate homologs of the genes in the Notch signaling pathway have been identified, and they have roles in almost every tissue and every stage of development. In many cases, novel roles (as illustrated by the role of Notch in somitogenesis) have been identified in vertebrates, with no direct or obvious counterparts in Drosophila development. Discoveries in invertebrate model systems and vertebrate model systems now play synergistic roles in our understanding of development. In the case of *mind bomb,* the gene was first identified by forward genetics in zebrafish (Itoh et al., 2003). Nevertheless, the analysis of *mind bomb* function in vertebrates relied heavily on models that were proposed for the functionally related E3 ligase Neuralized in Drosophila.

The goal of this chapter was to discuss Notch's versatile and diverse role in cell-fate specification and to illustrate that understanding its role in the fine patterning of cell fate requires an appreciation of how Notch function is integrated with other signaling mechanisms. There is now a growing recognition that, although Notch can influence tissue patterning through its influence on cell fate at a transcriptional level, it may simultaneously influence the organization of factors that determine cell morphology. Further integration of the role of Notch in cell fate and tissue morphogenesis remains an important and exciting challenge for the future.

SUMMARY

- The Notch receptor has an extracellular domain (NECD) that is bound noncovalently to a membrane-tethered intracellular fragment (NICD) that can function as a transcriptional activator. Activation is triggered by mechanisms that remove the NECD and by subsequent proteolytic

cleavages that release the NICD fragment into the nucleus, where, in association with CSL proteins and additional coactivator factors, it drives target gene expression. In the absence of NICD, CSL proteins associate with corepressors to maintain the basal repression of target genes.

- Notch interacts with DSL ligands that are also transmembrane proteins. Ubiquitylation serves as a signal for the internalization of DSL ligands. Endocytosis of the DSL ligands activates Notch, probably by helping to remove the NECD. The recycling of DSL ligands is also critical for their function. Factors that regulate DSL ligand and Notch endocytosis, recycling, and degradation play a critical role in biasing the outcome of Notch signaling.
- DSL ligands interact with Notch in neighboring cells (in trans) to activate the receptor. DSL ligands can also interact with Notch within the same cell (in cis). This interferes both with Notch activation by ligands in the neighboring cell and with the ability of the DSL ligand to effectively deliver a signal to neighboring cells.
- When DSL ligands activate Notch in neighboring cells, the outcome of the interaction depends on whether activation of Notch provides negative or positive feedback on DSL ligand function in the signal-receiving cell. The outcome also depends on which cells are capable of sending and receiving DSL signals.
- During lateral inhibition, Notch activation decreases the function of DSL ligands in the signal-receiving cell, thus making it harder for the cell to signal back to its neighbor. Reiterative interactions between competing cells can amplify small differences in signaling potential, and they can facilitate the selection of a single cell from a cluster of cells with similar potential. Central cells are selected by lateral inhibition when it operates in the context of an additional central biasing mechanism.
- When the activation of Notch results in positive feedback on DSL ligand function, mutually reinforcing interactions can help establish a signaling center that is dependent on high Notch activation.
- In some instances, the ability to send and receive DSL signals is restricted to complementary populations of cells. In this context, "inductive" Notch signaling from one population of cells helps specify fate in an adjacent population of cells.
- Some HER genes encode repressors that can inhibit their own transcription. During somitogenesis, a delay in feedback inhibition results in oscillating HER gene expression. Delta–Notch interactions between cells synchronize oscillations in the presomitic mesoderm. The oscillations stop when FGF signaling falls below a certain threshold, and the resulting locked periodic pattern of Notch activation is interpreted to define prospective boundaries in the presomitic mesoderm.

GLOSSARY

Ankyrin repeats
The tandemly repeated modules of about 33 amino acids. The repeat has been found in proteins of diverse function. The ankyrin fold appears to be defined by its structure rather than its function, because there is no specific sequence or structure that is universally recognized by it. (Definition adapted from Pfam 20.0.)

Epidermal-growth-factor–like repeats
Each epidermal-growth-factor–like repeat is made up of approximately 40 amino acids, and its structure is defined largely by six conserved cysteine residues that form three conserved disulfide bonds. Notch1 has 36 of these repeats.

Lateral inhibition
A cell–cell interaction in which a cell that is adopting a particular fate interferes with the ability of neighboring cells to adopt the same fate.

Lateral induction
A cell–cell interaction in which a cell delivers a signal to its neighbor to specify a particular fate.

RING domain
The RING (really interesting new gene) finger is a specialized type of Zn-finger that consists of 40 to 60 residues that bind two atoms of zinc and that is involved in mediating protein–protein interactions. The presence of a RING finger domain is a characteristic of RING-class E3 ubiquitin protein ligases that are capable of transferring ubiquitin from an E2 enzyme to a substrate protein.

Ubiquitylation
A multistep process that results in the covalent conjugation of a 76 amino acid polypeptide, Ubiquitin, to a lysine on a substrate protein. This modification, which is also called *ubiquitination* or *ubiquitinylation,* can alter the function of a protein in many ways, such as by targeting it for degradation in proteosomes, for endocytosis from the cell surface, or for internalization from the limiting membrane of late endosomes in vesicles to form multivesicular bodies or by changing the cellular distribution of a protein in other ways.

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RECOMMENDED RESOURCES

Notch Signaling: Definitions and Much More from Answers.com:
http://www.answers.com/topic/notch-signaling.
Vulval Development:
http://www.wormbook.org/chapters/www_vulvaldev/vulvaldev.html.
Memorial Sloan-Kettering Cancer Center: Notch Signaling:
http://www.mskcc.org/mskcc/html/53001.cfm.
Interactive Fly, Drosophila:
http://flybase.bio.indiana.edu/allied-data/lk/interactive-fly/neural/notch1.htm.

16 MULTIPLE ROLES OF T-BOX GENES

L. A. NAICHE and VIRGINIA E. PAPAIOANNOU

Department of Genetics and Development, College of Physicians and Surgeons of Columbia University, Columbia University, New York, NY

INTRODUCTION

The T-box genes are an evolutionarily ancient family of transcription factors defined by a shared region of sequence homology, the T-box, that encodes a DNA-binding domain. These genes are expressed dynamically in a variety of tissues throughout embryonic development. Mutations in T-box genes frequently result in developmental abnormalities in a wide range of species, and heterozygous or homozygous loss of T-box genes causes a number of human developmental syndromes.

Mutations have been made in a number of T-box genes in the mouse. The resulting phenotypes closely resemble the human syndromes caused by mutations in the orthologous human T-box genes, and have therefore provided us with model systems in which to investigate both the functions of T-box genes and the etiology of human developmental disorders. In this chapter, we discuss the effects of spontaneous T-box mutations in human, their corresponding engineered mouse mutations, and how both of these systems can be used to reveal novel information about this important gene family in particular and embryonic development in general.

I. T-BOX GENES: TRANSCRIPTION FACTOR GENES WITH MANY DEVELOPMENTAL ROLES

In 1927, a mouse gene discovered in a mutagenesis screen captured the interest of geneticists and developmental biologists. During the succeeding decades, it gathered many claims to fame. This gene, named *Brachyury* or *T* for tail, was recognized by its heterozygous mutant phenotype, a short tail, and it was lethal when homozygous, making it one of the earliest known examples of a

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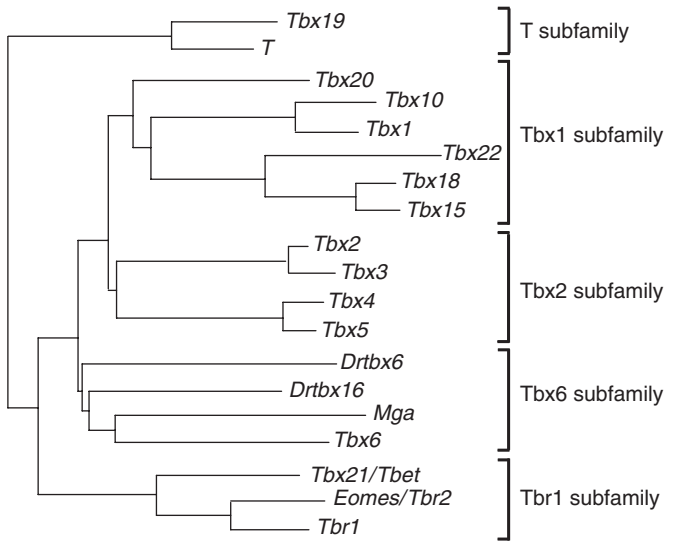

FIGURE 16.1 Schematic phylogenetic tree of the T-box gene family of vertebrates based on the phylogenetic analysis described by Papaioannou and Goldin (2004) showing the relationship of genes in the five subfamilies, which are indicated by brackets on the right. All genes are present in mammals, except the zebrafish genes *Drtbx6* and *Drtbx16,* which do not have orthologs in mammals. (Reprinted with permission from the *Annual Review of Genetics,* Volume 39, © 2005 by Annual Reviews [www.annualreviews.org; Naiche et al., 2005].)

developmental gene causing embryonic lethality (Dobrovolskaïa-Zavadskaïa, 1927*)*. *Brachyury* was the first mammalian gene to be positionally cloned, and, when the sequence of the gene and the structure of the protein were studied, it was found to code for a protein with a novel DNA binding motif called the T domain (Herrmann, 1995). Shortly after its cloning, *Brachyury* was recognized as the proband of a previously unknown family of transcription factor genes, now called the T-box gene family, that is present in all metazoans. Thus, although *Brachyury* has been the subject of intense study for more than half a century, other members of the T-box gene family are relative newcomers. Nonetheless, the T-box genes are essential for all manner of developmental processes, with multiple sites of expression and multiple roles in a wide variety of tissues (Naiche et al., 2005; Papaioannou, 2001).

The T-box genes can be grouped phylogenetically on the basis of sequence comparisons of the region coding for the T domain, thus providing a family tree that reflects the evolution of the genes (Papaioannou and Goldin, 2004). In mammals, there are 17 genes that are organized into five subfamilies (Figure 16.1). Although their phylogenetic relationships and their relationship to orthologs in other species are fairly clear, we still know relatively little about how the functions of T-box genes have evolved, because even very closely related family members have divergent functions. For example, *Tbx5* has a critical role in heart development, whereas the closely related *Tbx4* is not expressed there and has no role in the heart. What is clear is that T-box proteins function as transcription factors in a wide variety of developmental settings and that mutations in the genes are responsible for many different developmental abnormalities. Many T-box genes display eterozygous

phenotypes, and thus mutations in these genes have been readily recognized as being responsible for human developmental syndromes.

In this chapter, we will explore some of the multiple roles of T-box genes in developmental processes through an examination of human developmental syndromes caused by their mutations and the corresponding mouse mutations that model these syndromes. With the exception of cleft palate with ankyloglossia (Online Mendelian Inheritance in Man [OMIM] reference #303400), all of the human syndromes caused by mutations in T-box genes have counterparts in mouse models. We will consider how the study of the mouse models has led to the understanding of the basis of the human syndromes and how the variety of mutations in both human and mouse have led to a better understanding of gene function. We will consider genetic interactions between different T-box genes and the potential for interactions through common target genes in areas of expression overlap. The current state of the field emphasizes the need for conditional alleles and multiple allelic series to fully explore all sites of expression and the function of members of this important gene family, including those for which no human mutations have yet been discovered (Naiche et al., 2005; Papaioannou, 2001).

II. DNA BINDING AND TRANSCRIPTIONAL REGULATION BY T-BOX PROTEINS

The T-box DNA binding motif that characterizes all T-box proteins binds DNA in a sequence-specific manner. A palindromic DNA consensus sequence with high affinity for *Brachyury* protein was first defined and called the T-box binding element (TBE; Kispert and Herrmann, 1993). The Brachyury protein binds this sequence as a dimer, with each monomer binding a half site called a T-half site (5′-AGGTGTGAAATT-3′). The crystal structures of both Brachyury and TBX3 T-domain homodimers bound to the canonical TBE show that, despite differences in ternary structure, both T-box proteins make the same DNA contacts with the same amino acids, which indicates strong conservation of the underlying DNA binding functions (Coll et al., 2002; Muller and Herrmann, 1997). Work with other T-box proteins indicates they are all capable of binding the T-half site, although some have different optimal target sequences (e.g., Ghosh et al., 2001; Lingbeek et al., 2002; Sinha et al., 2000), and furthermore, different proteins have preferences for different combinations of T-half sites varying with regard to orientation, number, and spacing (Conlon et al., 2001; Sinha et al., 2000). In the context of the promoters of downstream target genes, these differences between T-box proteins in structure, binding preferences, and affinity may constitute the basis for target gene specificity, and they may be the basis for interactions among different T-box proteins in areas of expression overlap.

It is not known whether T-box proteins form functional heterodimers with other T-box family members, but there is evidence that T-box proteins function in combination with other transcription factors, such as homeodomain and GATA zinc finger proteins, to cooperatively bind promoters and synergistically upregulate target gene expression (see Naiche et al., 2005, for review). These interactions are highly specific and can add a further layer of target promoter specificity.

T-box proteins act as both activators and repressors of transcription of downstream genes. Activation domains have been mapped to the C-terminal

domains of several T-box proteins (Kispert, 1995; Stennard et al., 2003; Zaragoza et al., 2004), and Tbx2 and Tbx3 have been shown to repress transcription (Carreira et al., 1998; Habets et al., 2002; Lingbeek et al., 2002). Some T-box genes contain both activation and repression domains in their C-terminal domains, and Tbx2 has been reported to act in either fashion, depending on promoter context (Kispert, 1995; Paxton et al., 2002; Stennard et al., 2003). This type of versatility adds yet another layer of complexity to the repertoire of this gene family.

III. HUMAN SYNDROMES AND MOUSE MODELS

A. *TBX3* and the Ulnar Mammary Syndrome

The ulnar mammary syndrome (UMS) in humans (OMIM #181450) is an autosomal dominant disorder originally mapped to 12q23–q24.1, a region that contains both *TBX3* and *TBX5*. The syndrome was named for two of the more common abnormalities that make up this highly variable disorder: defects in the posterior part of the hand and arm (particularly the ulna) and a deficiency of mammary gland development, although not all individuals with UMS show both features. Other features are variably present in UMS, including a lack of apocrine glands that results in a lack of axial sweating, a lack of axial hair, delayed puberty in males, a variety of anomalies of the internal and external urogenital system, obesity, abnormalities of teeth and palate, laryngeal stenosis, ventricular septal defects, and posterior foot defects. The availability of large multigenerational kindreds with UMS allowed for the fine mapping of the disorder, and causative heterozygous mutations in *TBX3* were eventually discovered (Bamshad et al., 1997).

Within each kindred, family members show different abnormalities, thus illustrating the highly variable phenotype even in individuals with the same mutation. Additionally, in a study of UMS kindreds with 10 different *TBX3* mutations scattered throughout the gene, no correlation was found between the frequency or nature of different defects and mutations in specific parts of the gene. All kindreds had the same wide range of developmental abnormalities, whether the mutations were predicted to be loss-of-function or dominant-negative alleles (Bamshad et al., 1999). This suggests that all of these mutations result in haploinsufficiency and that interacting genes and/or epigenetic factors are responsible for the variability in phenotype. No homozygous mutant *TBX3* individuals have been identified.

Concurrent studies of the mouse *Tbx3* gene provided an explanation for the wide range of structures affected in cases of UMS. Like many T-box genes, *Tbx3* is expressed in a highly dynamic pattern throughout development. Notably, it is present in the margins of the developing limbs, in the mammary epithelium and mesenchyme, and in other tissues, such as the craniofacial region, the heart, the kidney, the genital papilla, the pituitary gland, the dorsal root ganglia, the yolk sac, and the lung (Chapman et al., 1996). In other words, expression is found in the embryonic precursors of many—if not all—of the tissues affected in UMS as well as in a number of other tissues not known to be affected in patients with UMS.

The mouse model for UMS is a null mutation in *Tbx3* that results in a loss of function by deleting part of the DNA binding domain (Davenport et al.,

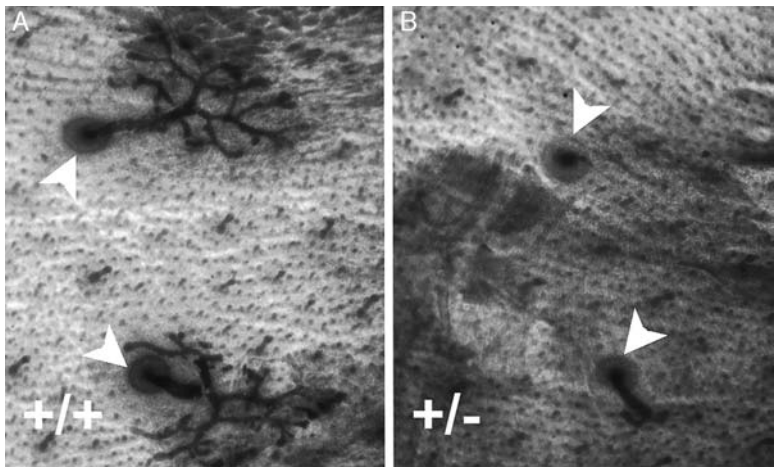

FIGURE 16.2 Mammary gland hypoplasia in *Tbx3* heterozygous mice. Carmine alum stain of the isolated skin showing the second and third mammary glands of late-gestation female mouse embryos, either **A**, wild type or **B**, heterozygous for a null mutation in *Tbx3*. Wild-type embryos have arborized ductal trees attached to a nipple (arrowheads), whereas a high proportion of heterozygous mutants have only a small duct with one or no branches and sometimes lack the nipple. Double heterozygosity for *Tbx2* and *Tbx3* exacerbates this defect, resulting in a higher proportion of animals with nipple and duct hypoplasia. (Adapted and reprinted with permission from Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc., from Jerome-Majewska et al., *Developmental Dynamics* 234:922, © 2005 Wiley-Liss, Inc. See color insert.)

2003). Mice homozygous for this mutation die at variable times during gestation, with multiple abnormalities. Some homozygous mutants die early as a result of abnormal cell death in the yolk sac and deficient vitelline circulation, but others have normal circulation and survive to late gestation, when they die of unknown causes. In addition to the life-threatening and sometimes lethal yolk sac deficiency, these homozygous mutant embryos display defects that are characteristic of UMS, such as ulnar and fibular ray deficiencies, a lack of mammary gland induction, and heart defects (Davenport et al., 2003, and unpublished observations). Although the mutant phenotype is generally more severe in the homozygous mice than in heterozygous humans, the defects are of a similar type to those seen in UMS. However, the homozygous mice reveal a possible human–mouse species difference in the relative severity of the effect in the forelimbs as compared with the hindlimbs. In UMS, the arms or hands are always affected, but the feet are only rarely affected; in *Tbx3* homozygous mutant mice, the hindlimbs are always affected, and they are affected much more severely than the forelimbs. Forelimbs have a variable loss of posterior digits and shortening or loss of the ulna, whereas the hindlimbs are severely truncated with the loss of all but the first digit. Molecular studies of limb development in *Tbx3* mutant mice reveal that the posterior defects are coincident with a downregulation of *Sonic hedgehog* (*Shh*) signaling in the posterior of the limbs, suggesting that *Tbx3* functions in the sonic hedgehog *Shh* pathway to establish posterior patterning and proliferation in the limb. Alternatively, *Tbx3* may be required to maintain cell proliferation in the *Shh*-expressing posterior limb tissue (Davenport et al., 2003).

Tbx3 heterozygous mice, on the other hand, which are genetically analogous to patients with UMS, are viable and fertile. They show no signs of either

forelimb or hindlimb abnormalities, although there are mild structural abnormalities in the external genitalia of adult females. They do, however, provide a good model for the mammary gland hypoplasia that is characteristic of UMS. Heterozygous females show a variable lack of development of the first and second pairs of mammary glands, and those glands that do form are hypoplastic, thereby mirroring the nipple and mammary defects seen in patients with UMS (Figure 16.2; Jerome-Majewska et al., 2005). Furthermore, the availability of a mouse mutation in *Tbx2* (Harrelson et al., 2004), which is a closely related T-box gene that is coexpressed in the mammary mesenchyme, led to the discovery of an interaction between *Tbx3* and *Tbx2* in mammary gland induction in which double heterozygotes have a more severe mammary gland reduction than single *Tbx3* heterozygotes (Jerome-Majewska et al., 2005).

This observation of a mammary gland interaction between *Tbx2* and *Tbx3* may provide a link between the mammary developmental hypoplasia in UMS and the hyperplasia of mammary tumors. *TBX2* is amplified and overexpressed in a large fraction of *BRCA1*- and *BRCA2*-related breast tumors (Sinclair et al., 2002), and both *TBX2* and *TBX3* have been molecularly linked to the cell cycle machinery via the ARF-MDM2-p53 pathway in cell lines, although not in fetal or prepubertal mouse development (Brummelkamp et al., 2002; Jacobs et al., 2000; Jerome-Majewska et al., 2005). Further work, including the production of over- and misexpression alleles in mice, will be required to determine how these genes contribute to normal mammary development and how misregulation can contribute to mammary tumorigenesis.

The mouse model of UMS, despite revealing species differences in dose sensitivity to haploinsufficiency and species differences in the penetrance of the phenotype in the limbs, provides valuable information and material for the study of UMS. Studies in the mouse confirm the inherently variable nature of the effect of loss of *Tbx3*, because the mild-to-severe range of defects were observed in genetically identical inbred strains that eliminate the effects of variable genetic backgrounds (Davenport et al., 2003; Jerome-Majewska et al., 2005). By virtue of the greater severity of defects in the homozygous mutants, the mouse model for UMS has revealed additional sites of *Tbx3* activity not known or observed in UMS patients, such as the yolk sac. The mouse model also demonstrates that, within a species, different structures differ in their sensitivity to haploinsufficiency; mammary gland and genital defects are observed in heterozygous mice, but limb and yolk sac problems are seen only in homozygotes. For further exploration of these issues, the mouse model system can be used to create hypomorphic alleles and overexpression transgenes to manipulate *Tbx3* levels both upward and downward, conditional alleles to allow for tissue-specific ablation of gene function at specific times in development, and specific deletions of sub-domains of the gene to allow for further investigation of the functional domains of the *Tbx3* protein. These mutations can then either be tested on uniform genetic backgrounds or examined in variable genetic backgrounds to look for modifiers of the phenotype.

B. *TBX1* and the DiGeorge Syndrome

A convergence of research on T-box genes in mouse and a number of microdeletion syndromes in humans collectively known as 22q11.2 deletion syndrome

(22q11.2DS; OMIM #188400) resulted in the recognition of *TBX1* as a major factor in the etiology of this syndrome. Variously described as DiGeorge syndrome, velocardiofacial syndrome, Shprintzen syndrome, and conotruncal face syndrome, 22q11.2DS is characterized by cardiovascular anomalies, including outflow tract and aortic arch defects; craniofacial anomalies, including cleft palate; ear defects; thymic and parathyroid hypoplasia or aplasia; skeletal anomalies; kidney abnormalities; and neurobehavioral problems. Most commonly, the syndrome results from a 3 megabase pair (Mbp) deletion on chromosome 22 that includes more than 30 genes or, less commonly, from a 1.5 Mbp deletion that includes about 20 genes; both deletions include *TBX1*. The presence of repeated DNA sequences, called low-copy repeats, at the deletion breakpoints are thought to lead to a high rate of interchromosomal recombination and deletion (Saitta et al., 2004), thereby making this syndrome one of the most common birth defects (approximately 1 in 4000 live births). Despite the potential involvement of a large number of genes, several lines of evidence have implicated haploinsufficiency for *TBX1* as the major genetic cause of the syndrome, including the discovery of *TBX1* mutations in a few patients with the syndrome but without microdeletions (Gong et al., 2001; Yagi et al., 2003).

Although a minimal critical region for 22q11.2DS cannot be pinpointed by an analysis of overlapping deletions, *TBX1* is one of the candidate genes in the commonly deleted region. Chromosomal engineering to produce a deletion of the syntenic region in the mouse (which includes mouse *Tbx1*) provided a haploinsufficiency model that recapitulated some of the features of 22q11.2DS (Lindsay et al., 1999), including learning and memory impairments (Paylor et al., 2001). *Tbx1* has a developmental expression pattern that places the gene product in a prime position to affect pharyngeal, heart, ear, and skeletal development. It is expressed in the core mesoderm of the pharyngeal arches, the pharyngeal endoderm, the otic placode, the head mesen-

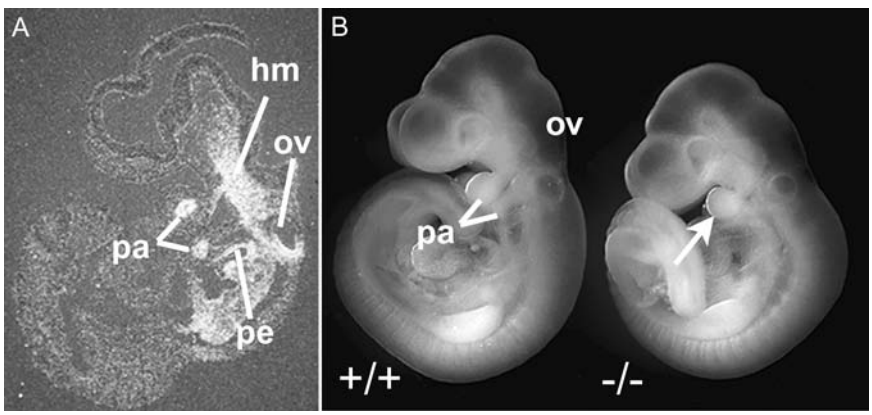

FIGURE 16.3 **A, Expression of *Tbx1* in a parasagittal section showing expression (white signal) in the otic vesicle (ov), the core of the pharyngeal arches (pa), the pharyngeal endoderm (pe), and the head mesenchyme (hm). B,** Whole mount wild-type (+/+) and *Tbx1* mutant (−/−) mid gestation embryos. The mutant embryo has only one pharyngeal arch (arrow) and a small otic vesicle. (**A** adapted and reprinted with permission from Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc., from Chapman et al., *Developmental Dynamics* 206:379 ©1996 Wiley-Liss, Inc.; **B** adapted and reprinted with permission from Macmillan Publishers Ltd. from Jerome and Papaioannou, *Nature Genetics* 27:286 © 2001. See color insert.)

chyme, the tooth buds, the lung epithelium, and the developing vertebrae, which are areas that encompass the precursors of virtually all of the tissues affected in 22q11.2DS patients (Figure 16.3; Chapman et al., 1996).

Loss-of-function mutations in mouse *Tbx1* strongly support the contention that *TBX1* is causative of 22q11.2DS, because heterozygotes demonstrate haploinsufficiency defects in thymus and aortic arch artery development, and homozygotes show a full spectrum of phenotypic abnormalities typical of 22q11.2DS, albeit with more severe phenotypes (Jerome and Papaioannou, 2001; Kelly et al., 2004; Lindsay et al., 2001; Merscher et al., 2001). Furthermore, patients with *TBX1* mutations display the major phenotypes associated with 22q11.2DS, with the notable exception of mental retardation (Yagi et al., 2003), which is a common finding in patients with the 22q11.2 microdeletion. Two of the *TBX1* mutations result in amino acid substitutions in highly conserved regions within the DNA binding domain of the protein, and the third results in a truncated protein with an intact DNA binding domain but no C-terminus. The first two mutations very likely interfere with DNA binding or dimerization, whereas the third deletes a nuclear localization signal and destroys the transcriptional activity of the protein (Stoller and Epstein, 2005).

Even with this strong evidence from the mouse and human models that mutations in *TBX1* underlie most of the defects in 22q11.2DS, there are a number of issues related to the fact that the vast majority of patients also have a large number of other genes deleted that could contribute to the phenotype, either as a contiguous gene syndrome or by regulating *TBX1*. Several other genes within the commonly deleted region are expressed in the precursors of affected tissues, thus leading to the possibility that their haploinsufficiency may contribute directly to some aspects of the syndrome. The variability of the syndrome could result from genetic background effects, but it could also be affected by the variable deletion of genes that can act as modifiers of *TBX1*. Some features, like mental retardation, are not obviously connected with any known function or expression pattern of *Tbx1* and could result from the deletion of other genes, as indicated by the absence of this feature in the patients with *TBX1* mutations. A variety of approaches used to explore these possibilities have led to a clarification of the consequences of *Tbx1* mutation.

Exploration of other 22q11.2DS candidate genes led to the discovery of an interaction between *Tbx1* and *Crkl,* the ortholog of which is located within the human 3 Mbp deletion. Mice homozygous for a null mutation in *Crkl,* which codes for a crk family adapter protein, phenocopy multiple aspects of 22q11.2DS, whereas heterozygous mutants show only rare craniofacial and thymic anomalies. Double heterozygotes for *Crkl* and *Tbx1* show a greatly increased penetrance of aortic arch, thymic, and parathyroid defects as compared with either type of single heterozygotes (Guris et al., 2006; Guris et al., 2001; Jerome and Papaioannou, 2001), which indicates that a genetic interaction between these two genes affects the development of the pharyngeal apparatus. Defects in the pharyngeal region of double heterozygotes are associated with functional aberrations in two major signaling pathways: retinoic acid (RA) and fibroblast growth factor (FGF) signaling. Triple heterozygous embryos with mutations in *Crlk, Tbx1,* and *Raldh2* (a gene that encodes a key enzyme in the synthesis of RA) show an amelioration of the phenotype, which indicates that the gain of function of RA seen in double heterozygotes

is at least partially responsible for the double heterozygous phenotype. Similarly, in triple heterozygotes with *Crlk, Tbx1,* and *Fgf8* mutations, the reduction of *Fgf8* exacerbates the severity of the double heterozygous phenotype (Guris et al., 2006), thereby further confirming a link between *Tbx1* and FGF signaling and its importance in 22q11.2DS (Baldini, 2005).

The importance of the 22q11.2DS in human medicine has led to a large research effort concentrated on the *Tbx1* gene and dissecting its role in the development of different organs. This has resulted in the production of a number of *Tbx1* alleles. Conditional alleles have been used to determine the requirement for *Tbx1* in different tissues and at different times in development. Studies have highlighted the multiple roles of *Tbx1* and have indicated that a loss of expression in the pharyngeal endoderm alone can account for most of the pharyngeal abnormalities, although the gene is expressed in other pharyngeal tissues (Arnold et al., 2006; Xu et al., 2005; Xu et al., 2004). An expression reporter allele (Lindsay et al., 2001) has been useful for detecting low levels of gene expression with high sensitivity. Hypomorphic alleles and overexpression transgenes have been used to investigate the effect of altered gene dosage (Liao et al., 2004; Xu et al., 2004), and a lineage-tracing allele has revealed the fate of *Tbx1*-expressing cells (Lindsay et al., 2001; Xu et al., 2005; Xu et al., 2004; see also Baldini, 2005, and Yamagishi and Srivastava, 2003, for reviews).

With this considerable body of knowledge about the developmental effects of mutations in *Tbx1,* there is still a paucity of information about the transcriptional targets of the gene. To date, only one direct target, *Fgf10,* has been confirmed (Xu et al., 2004), but the multiple tissues affected indicate that the battery of downstream targets is likely to be large, thus providing a fertile area for future research.

C. *TBX5* and Holt–Oram Syndrome

Holt–Oram syndrome (HOS; OMIM #142900) is a developmental syndrome that affects heart and arm development and is apparent in approximately 1 in 100,000 births. Individuals with HOS show a range of defects, including both atrial and ventricular septal defects, problems with cardiac conduction, and other heart abnormalities, as well as forelimb defects ranging from minor thumb elongation to severe arm truncation. Physical mapping in affected families linked this disorder to chromosome 12q2, after which a candidate gene approach identified *TBX5* as the causative gene (Basson et al., 1997; Li et al., 1997). Since the original reports, at least 10 new reports of HOS cases comprising several dozen novel *TBX5* mutations have been published, leading to the establishment of an online database of known *TBX5* mutations (http://www.uni-leipzig.de/~genetik/TBX5; Heinritz et al., 2005). In addition to the large cohort of HOS patients with *TBX5* mutations, the profound effect of *TBX5* function on heart development is also illustrated by a study of patients with nonsyndromic septal defects in which mutations in *TBX5* were found in nine out of 68 hearts but not in peripheral blood (Reamon-Buettner and Borlak, 2004). This suggests that somatic mutation of *TBX5* is a relatively common cause of heart malformations.

Expression studies in mouse and chick reveal that *Tbx5* is expressed in both the heart and the forelimb from their inceptions through late development. When the *Tbx5* mutation was made in the mouse, mutants were found

to have severe malformations of the heart (Bruneau et al., 2001). Heterozygous mouse mutants closely recapitulate the HOS heart phenotype, with frequent atrial septal defects, abnormalities of the conduction system, and occasional ventricular septal defects. Several genes involved in heart development, such as *connexin40* and *ANF,* are downregulated in the *Tbx5* heterozygotes, thus demonstrating that these genes are highly sensitive to *Tbx5* dosage. *Tbx5* homozygous mutant embryos have an even more dramatic phenotype in which the heart is severely hypoplastic, does not loop, completely fails to express *ANF,* and has dramatically lower levels of an array of cardiac differentiation markers. The *Tbx5* homozygous mutant heart does nonetheless express cardiac-specific transcription factors and contractile proteins, which indicates that *Tbx5* is required for cardiac differentiation but not specification.

The mouse *Tbx5* mutation also revealed the importance of *Tbx5* in forelimb development. Mice that are heterozygous for *Tbx5* have hypoplastic wrist bones and elongated phalanges of the first forelimb digit (thumb), which is similar to what has been reported in some HOS patients. In *Tbx5* null embryos, the forelimb bud completely fails to form. This was shown to be caused by the absence of *Fgf10,* a key gene in limb initiation and outgrowth in the forelimb field (Ahn et al., 2002). This is probably a direct effect, as *Tbx5* can directly regulate the *Fgf10* promoter (e.g., Ahn et al., 2002). The forelimb abnormalities seen in HOS patients may be the result of a partial loss of *Fgf10* function, although this remains to be shown.

In general, the mouse model of a human disorder provides opportunities to investigate the molecular function of a target gene with a variety of techniques that are unavailable for use in human patients (e.g., *in situ* hybridization, tissue or organ explants). In an unusual twist, the large number of *TBX5* mutations found in HOS patients have provided a wealth of data regarding the molecular function of *Tbx5* comparable to that provided by the mouse model. Comparison of the severity of limb defects with the severity of heart defects in human pedigrees with mutations in different parts of *TBX5* has provided suggestions regarding which protein domains are key to the phenotypes in each organ (Basson et al., 1999), although these relationships have not held up in all analyses (Brassington et al., 2003). Several point mutations in *TBX5* have been found to cause HOS, which has allowed investigators to compare normal and disease-causing variants in biochemical assays to ascertain the mechanistic nature of the causative defect. Not surprisingly, many disease-causing point mutations simply ablate *TBX5* DNA binding or activation domains. More remarkably, mutations in which *TBX5* protein binds DNA successfully but is not capable of interaction with the homeobox transcription factor NKX2.5 or the coactivator protein TAZ also cause disease, which shows that transcriptional cofactors are key components of T-box transcriptional function (Fan et al., 2003; Murakami et al., 2005). It has since been shown that *GATA4, NKX2.5,* and *TBX5* synergistically upregulate target genes *Nppa*, *connexin40,* and *p204*, and point mutations in any of these three genes that affect only their mutual interaction are sufficient to cause heart malformations (Ding et al., 2006; Fan et al., 2003; Garg et al., 2003; Hiroi et al., 2001; Linhares et al., 2004).

Mutations in *TBX5* have not been found in all individuals diagnosed with HOS. In some cases, this may be because of causative mutations in regulatory

regions of *TBX5,* which are not normally sequenced. In others cases, individuals have mutations in other genes that produce the same phenotype, which suggests that they may be in the same molecular pathway as *TBX5*. Mutations in *SALL4,* which is normally associated with Okihiro syndrome (OMIM #607323), are sometimes misdiagnosed as HOS (Kohlhase et al., 2003). Mouse *Sall4* has recently been shown to be a downstream target of *Tbx5* in both the forelimb and the heart (Harvey and Logan, 2006; Koshiba-Takeuchi et al., 2006).

D. *TBX4* and the Small Patella Syndrome

Unlike other T-box genes in this review, the original mouse mutation of *Tbx4* was made before a corresponding human syndrome had been proposed. *Tbx4* is principally expressed in two regions of the mouse embryo: the hindlimb and the allantois. The allantois forms the umbilical cord, which is vital for embryonic survival in placental mammals, and *Tbx4* null embryos die mid-gestation as a result of the failure of umbilical formation (Naiche and Papaioannou, 2003).

Tbx4 is closely related to *Tbx5,* and the expression of *Tbx4* in the hindlimb is complementary to that of *Tbx5* in the forelimb, thus leading to the hypothesis that these two genes perform similar functions in their respective limbs. However, unlike the *Tbx5* null forelimb, the *Tbx4* null hindlimb initially expresses *Fgf10* and forms a morphologically visible bud. The hindlimb bud fails to maintain *Fgf10* and does not progress beyond the bud stage, which suggests that *Tbx4* may be required in the feedback loop that normally maintains *Fgf10* expression (Naiche and Papaioannou, 2003). The complementary expression of *Tbx4* and *Tbx5* in the hindlimb and the forelimb has also suggested that these genes control the differences between hindlimb and forelimb identity, but this idea has recently been discredited (Minguillon et al., 2005; Naiche and Papaioannou, 2007).

Recently, mutations in human *TBX4* have been linked to small patella syndrome (OMIM #147891; Bongers et al., 2004). In this rare syndrome, skeletal defects are variably present at all axial levels of the leg, including abnormal pelvic ossification, small or missing patella, and a large gap between the first and second toes. This suggests that *TBX4* is required for the correct development of all of the hindlimb elements. Some patients with Okihiro syndrome (patients with mutations in *SALL4*) also present with the enlarged gap between the first two toes that is characteristic of small patella syndrome, which suggests that *SALL4* may function downstream of both *TBX4* and *TBX5* in limb development (Kohlhase et al., 2003; Figure 16.4).

E. *TBX19* and Adrenocorticotrophic Hormone Deficiency

One of the predominant causes of early onset isolated adrenocorticotrophic hormone (ACTH) deficiency leading to adrenal insufficiency in infants (OMIM #201401) is a mutation of the *TBX19* gene, also known as *TPIT.* The absence of pituitary proopiomelanocortin (POMC), the biochemical precursor to ACTH, leads to adrenal insufficiency in these patients. Unlike other known human T-box related disorders, all eight loss-of-function alleles of *TPIT* that cause disease are recessive (Pulichino et al., 2003a). During embryonic development in the mouse, *Tbx19* has a very restricted expression pattern: it is seen only in two lineages of the pituitary gland (the corticotrophs

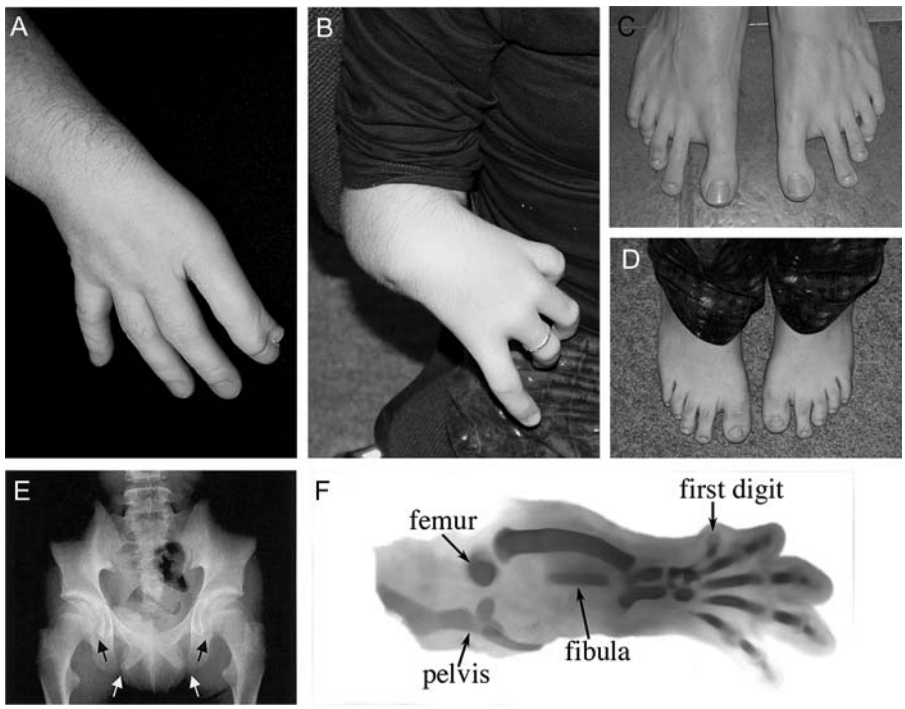

FIGURE 16.4 **Limb malformations caused by mutations in T-box gene pathways. A,** Holt–Oram patient with a mutation in *TBX5* exhibits both the loss and partial duplication of the anterior digits (He et al., 2004). **B,** Mutation of *SALL4,* a putative *TBX5* target, causes similar limb malformations in Okihiro syndrome (Kohlhase et al., 2003). **C,** Patients with small patella syndrome, caused by *TBX4* mutations, show a characteristic gap between the first two toes as well as short fourth and fifth toes. **D,** *SALL4* may also be a *TBX4* target, because this patient with Okihiro syndrome has similar foot abnormalities. **E,** Patients with small patella syndrome show abnormal ossification of the pelvis (arrows) as well as the foot defects in **C** and the knee defects for which the syndrome is named (Bongers et al., 2004). **F,** Mice in which *Tbx4* has been ablated immediately after limb development starts also show defects at all axial levels of the hindlimb, including a hypoplastic pelvis, femur, and fibula and the partial or complete loss of anterior digits (Naiche and Papaioannou, 2007). (**A** reprinted with permission from He et al., 2004. **B** and **D** reprinted with permission from the BMJ Publishing Group from Kohlhase et al., *J Med Genet* 40:473 ©2003. **C** and **E** reprinted with permission from the University of Chicago Press from Bongers et al., *Am J Hum Genet* 174:1239 ©2004 by the American Society of Human Genetics.)

of the anterior lobe and the melanotrophs of the intermediate lobe), and its expression starts before POMC expression. Like humans, mice that are heterozygous for loss-of-function *Tbx19* mutations have no ACTH deficiency, and they have normal numbers of POMC-expressing cells in their pituitaries, with no evidence of any haploinsufficiency effects (Pulichino et al., 2003a; 2003b). Homozygous mice, on the other hand, indicate that *Tbx19* is required for the terminal differentiation of corticotrophs and melanotrophs and for the upregulation of POMC. *Tbx19* activates POMC transcription in cooperation with another transcription factor, *Pitx1,* through the recruitment of coactivators to its cognate DNA target, the Tpit/Pitx regulatory element in the POMC promoter (Lamolet et al., 2001; Liu et al., 2001; Maira et al., 2003). *Tbx19* also represses gonadotroph differentiation, thus leading to the idea that it controls alternative cell fates during pituitary development (Lamolet et al., 2001; Liu et al., 2001; Pulichino et al., 2003b).

F. Loss of Multiple T-box Genes

Recently, a family has been identified in which a hemizygous deletion encompasses both *TBX3* and *TBX5* (Borozdin et al., 2006). The affected members of this family show symptoms of both HOS and UMS, characterized by the posterior limb defects and hypoplastic nipples typical of UMS and the anterior limb defects and heart abnormalities typical of HOS. Despite the fact that *TBX3* and *TBX5* are coexpressed in regions of the heart and limb, the loss of both genes did not appear to exacerbate the phenotype of either syndrome. In fact, the phenotypes of all three patients were fairly mild, leading the authors to speculate that the affects of the loss of *TBX3* (generally a transcriptional repressor) may ameliorate the effects of the loss of *TBX5* (generally a transcriptional activator). It is impossible to draw firm conclusions from a single family, but the combined UMS/HOS phenotype does indicate that there is no redundancy between *TBX3* and *TBX5* in functions that are sensitive to haploinsufficiency.

IV. FUTURE DIRECTIONS

Mutations in different T-box genes are a significant contributor to human malformation and disease, and they result in both common and rare developmental syndromes. The furthering of our understanding of this gene family will not only greatly enhance our understanding of the mechanisms of embryonic development, but it may also allow us to affect the outcomes of these disorders, particularly those that have postnatal outcomes, such as asthma or hormonal deficiencies. Advances in the sequencing and elucidation of HOS etiology have already allowed for genetic screening of *in vitro*-fertilized human oocytes and resulted in the birth of two healthy infants to parents with HOS (He et al., 2004).

However, much remains unknown about T-box gene function and regulation. A few target genes controlled by T-box protein transcriptional regulatory activity have been identified, but most remain elusive. Biochemical research has suggested that individual T-box proteins have different targets as a result of differential preferences for half-site spacing and binding partners, but little is known about how these factors play out *in vivo*. Mutations in individual T-box genes have shown defects in tissues where other T-box genes are expressed, thus indicating that different members of this family can play different roles even in the same cell. However, gene swaps have also shown that there can be functional redundancy between T-box genes (Minguillon et al., 2005). Isolated reports have indicated that T-box genes can regulate each other's expression in some tissues (e.g. Naiche and Papaioannou, 2003), but most aspects of the regulation of T-box gene expression remain uncharacterized.

Use of model systems is starting to address these issues. Conditional alleles are being developed to precisely ablate gene function to isolate the source of defects when multiple interacting affected tissues express the same T-box gene. Conditional alleles are also being used to bypass early defects and to examine the results of loss of gene function later in embryonic development. Over- and underexpression constructs are being used to create allelic series involving varying gene dosages in different tissues. Mutations in individual T-box genes are being combined by breeding to clarify specific and redundant effects of the loss of multiple genes from the same tissue. Mutant tissues

are being used in comparative microarrays and chromosome immunoprecipitation assays to isolate targets of T-box transcriptional regulation. All of these experiments should soon expand our knowledge of T-box gene function and of the developmental processes that they control.

SUMMARY

- The T-box gene family consists of 17 related genes that encode DNA binding proteins that function as transcription factors that are important in many developmental processes.
- Mutations in T-box genes result in congenital abnormalities, many of which have effects in heterozygous individuals, thus indicating dosage sensitivity.
- *TBX1* has been identified as a major factor in the DiGeorge deletion syndrome, and mouse mutations have allowed for the detailed study of the role of this gene in the development of the heart and the pharyngeal apparatus.
- Mutations in *TBX3, TBX5, TBX4,* and *TBX19* all result in human developmental syndromes (ulnar mammary, Holt–Oram, small patella, and isolated deficiency of ACTH, respectively), and mouse models are being used to explore the mechanism of action of these genes.
- Sophisticated genetic engineering in mice and the study of naturally occurring mutations in humans will continue to reveal the full range of developmental effects of T-box genes.

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GLOSSARY OF TERMS

Conditional allele
An allele that is engineered to function normally until it is recombined by the introduction of a recombinase to produce a mutant allele in a tissue- or temporal-specific manner.

Dominant negative
A mutated protein that interferes with the function of normal copies of the same protein.

Half-site
One half of the palindromic T-box binding element.

Haploinsufficiency
A situation in which a mutation reduces the level of a gene product below threshold levels and a phenotypic effect results.

Online Mendelian Inheritance in Man (OMIM)
A catalog of human genes and genetic disorders developed by the National Center for Biotechnology Information.

T-box binding element (TBE)
The palindromic DNA consensus sequence with a high affinity for binding the T domain.

T-box domain
Also called the T domain, it is the defining feature of the T-box gene family, and it consists of a conserved sequence encoding a polypeptide domain that extends across a region of 180 to 200 amino acid residues.

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Papaioannou VE, Silver LM: The T-box gene family, *Bioessays* 20:9–19, 1998.

RECOMMENDED WEB SITES

Online Mendelian Inheritance in Man (OMIM):
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi? db=OMIM
The Human TBX5 Gene Mutation Database:
http://www.uni-leipzig.de/~genetik/TBX5

III

MORPHOGENETIC AND CELL MOVEMENTS

17 GASTRULATION IN VERTEBRATES

LILIANNA SOLNICA-KREZEL and DIANE S. SEPICH

Department of Biological Sciences, Vanderbilt University, Nashville, TN

INTRODUCTION

The early inductive processes during vertebrate development specify precursors of the three germ layers—the endoderm, the mesoderm, and the ectoderm—on the surface of a spherical, flat, or cup-shaped blastula (Figure 17.1, I through L; Beddington and Smith, 1993; Kane and Warga, 2004; Keller and Shook, 2004; Stern, 2004; Tam and Gad, 2004). However, at the end of embryogenesis, a conserved body plan of vertebrate embryos features a rostrocaudally (head to tail) elongated axis (reviewed in Schoenwolf and Smith, 2000; Solnica-Krezel, 2005). Along the dorsal–ventral axis, the nervous system takes the most dorsal position above the notochord, which is flanked bilaterally by segmented somites. The most ventrally positioned are the alimentary structures, including the gut (Figure 17.1, M through P). This morphologic makeover is achieved by the process of gastrulation, which is named after the Greek word *gaster,* meaning "stomach" or "gut." Presently, the term *gastrulation* denotes a set of concurrent morphogenetic processes that transforms a rather unstructured early animal embryo into a gastrula with three germ layers that are shaped into the body plan typical for a given systematic group.

A. Gastrulation Movements as Tissue-Shaping Processes

Vertebrate gastrulation is accomplished by a combination of four evolutionarily conserved morphogenetic movements that can be classified on the basis of how they alter tissue shape (Keller and Davidson, 2004; Solnica-Krezel, 2005). Internalization (emboly) generates the three germ layers by bringing the prospective mesodermal and endodermal cells underneath the future ectoderm via a blastopore, which is the structure that is central to the process of gastrulation and that is also known as the *blastoderm margin* in fish and the *primitive streak* in amniotes. Epibolic movements spread and thin tissues during gastrulation. The concurrent convergence and extension (C&E) movements narrow tissues mediolaterally and elongate them from head to tail.

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B. Gastrulation Movements as Cellular Machines

These large-scale morphogenetic processes of gastrulation involve coordinated activities of cell populations (Keller and Davidson, 2004; Leptin, 2005). Such morphogenetic cell behaviors include directed cell migration and cell rearrangements of mesenchymal cells or cells forming epithelial sheets. Gastrulation movements can also involve the breaking of epithelial sheets into individual cells for the so-called epithelial-to-mesenchymal transition (EMT) as well as the reverse process of mesenchymal-to-epithelial transition. The EMT and the mesenchymal-to-epithelial transition are integral to many morphogenetic processes during animal development, and are discussed in other chapters of this book (see also Shook and Keller, 2003). Tissue morphogenesis can also be driven by cell proliferation, cell growth, or simple cell shape changes. The major gastrulation movements are accomplished by one or a combination of the different morphogenetic cell behaviors. Moreover, the repertoire of specific cell behaviors employed during a given gastrulation movement may vary among the vertebrate species.

I. VARIABLE MORPHOLOGY AND MORPHOGENETIC MOVEMENTS OF PREGASTRULATION EMBRYOS

The fertilized zygote possesses all of the instructions for its embryonic development encoded in the zygotic genome as well as in maternally deposited substances. The relative contributions of the zygotic and maternal control varies among vertebrates; this is reflected particularly in the speed and pattern of the early cleavages and, consequently, in the morphology of the blastula. The fish and amphibian embryos develop externally, and the fast rate of their early development ensures the swift formation of an independent larvae. These embryos rely on large amounts of energy stores in the yolk and on maternal determinants that mediate development until the 1000-cell stage, when the zygotic genome becomes transcriptionally active and takes over control (Kane and Kimmel, 1993; Newport and Kirschner, 1982). It is also after the midblastula transition when cells become motile in the zebrafish embryo (Kane and Kimmel, 1993). The abundance of yolk material is generally concentrated in the vegetal region of the zygote, and it is either distributed between the blastomeres via complete cleavages, as in frog embryos, or it is deposited in a separate yolk cell, as observed in incompletely cleaving fish blastulae (Figure 17.1, A and B). As a consequence, the fish blastula features a mound of blastomeres at the animal region on top of a large syncytial yolk cell. By contrast, the frog gastrula consists of smaller blastomeres at the animal hemisphere surrounding a blastocele cavity and larger blastomeres in the vegetal region (Figure 17.1, E and F).

The chick zygote, which is also endowed with a large yolk supply, engages in meroblastic cleavages to partition the cytoplasmic island into the centrally located small cells and larger, yolky, and open cells at the periphery (Figure 17.1, C). Further cellular divisions generate a superficial single-cell–thick epithelium, known as the *epiblast,* which will give rise to the embryo proper and which consists of a central area pellucida surrounded by the peripheral area opaca (Figure 17.1, G). Underlying the entire superficial layer is the primitive endodermal layer. Although the syncytial yolk cell of the fish

embryo and the primitive endodermal layer of the chick embryo will contribute only to become extraembryonic structures, they are thought to play important and active roles in embryo patterning (Stern, 2004).

Mammalian embryos develop within the resource-rich uterus. Consequently, their development is rather slow, and the amount of maternal energy and determinants deposited in the completely cleaving blastomeres is minimal (Figure 17.1, D). Likewise, the genetic control of embryogenesis is heavily shifted toward the zygotic genome, which becomes active already at the 2-cell stage (Kanka, 2003). The communication of the embryo with the mother's uterus is achieved via extraembryonic structures, the formation of which starts during the early cleavages. However, the mouse, human, and chick blastulae are similar in that future embryonic tissues form a single-cell–thick epithelium, which is flat in chick and human but shaped like a cup in the mouse (Figure 17.1, E through H). In the murine embryo, extraembryonic endoderm surrounds the cup on the outside, whereas extraembryonic ectoderm is positioned at its rim (see Figure 17.1, H). In essence, one way to picture mouse embryo morphology is to imagine a weight placed in the center of a flat chick embryo that bends it into a cup shape (Tam and Gad, 2004).

II. CONSERVED DISTRIBUTION OF TISSUE PROGENITORS AT THE ONSET OF VERTEBRATE GASTRULATION

Gastrulation is preceded by but also entails inductive processes that specify and pattern the germ layers, which are described in other parts of this book. As a result of significant maternal contribution in fish and frog eggs, the early inductive events in these embryos rely on asymmetrically distributed maternal determinants (Pelegri, 2003). Whereas the development of the mouse embryo is considered to be largely regulative, evidence for the existence of some early bias is accumulating (Zernicka-Goetz, 2006). During gastrulation, the patterning processes are primarily controlled by the Spemann–Mangold organizer (SMO), which is the key signaling center located in the axial aspect of the blastopore (Spemann, 1938). During the last two decades, extraordinary progress has been made in the elucidation of the molecular genetic mechanisms that govern the specification of embryonic polarity, germ layers, and the diversification of cell fates during the embryogenesis of both invertebrate and vertebrate embryos (De Robertis et al., 2000; Harland and Gerhart, 1997; Niehrs, 2004). As discussed in other chapters of this book, these studies reveal a remarkable conservation of developmental patterning mechanisms from invertebrate through vertebrate animals. For example, an activity gradient of bone morphogenetic proteins (BMPs) has been shown to specify the ventral/posterior to dorsal progression of cell fates in the germ layers in a fruit fly and in all vertebrate gastrulae (De Robertis and Kuroda, 2004; Hammerschmidt and Mullins, 2002). Such conservation of developmental mechanisms has been hinted at by the earlier work of embryologists on the basis of comparative anatomy (Schoenwolf and Smith, 2000; Tam and Quinlan, 1996).

However, the dynamic and complex nature of the morphogenetic processes of gastrulation made this process more difficult to elucidate. Moreover, the distinct architecture of early embryos, even among vertebrates, suggested that different cellular and molecular mechanisms were employed. This situation is

changing rapidly. New studies in many model systems are uncovering cellular behaviors that drive individual gastrulation movements, and genetic screens implicate unexpected genes in gastrulation. Delineating these genes is starting

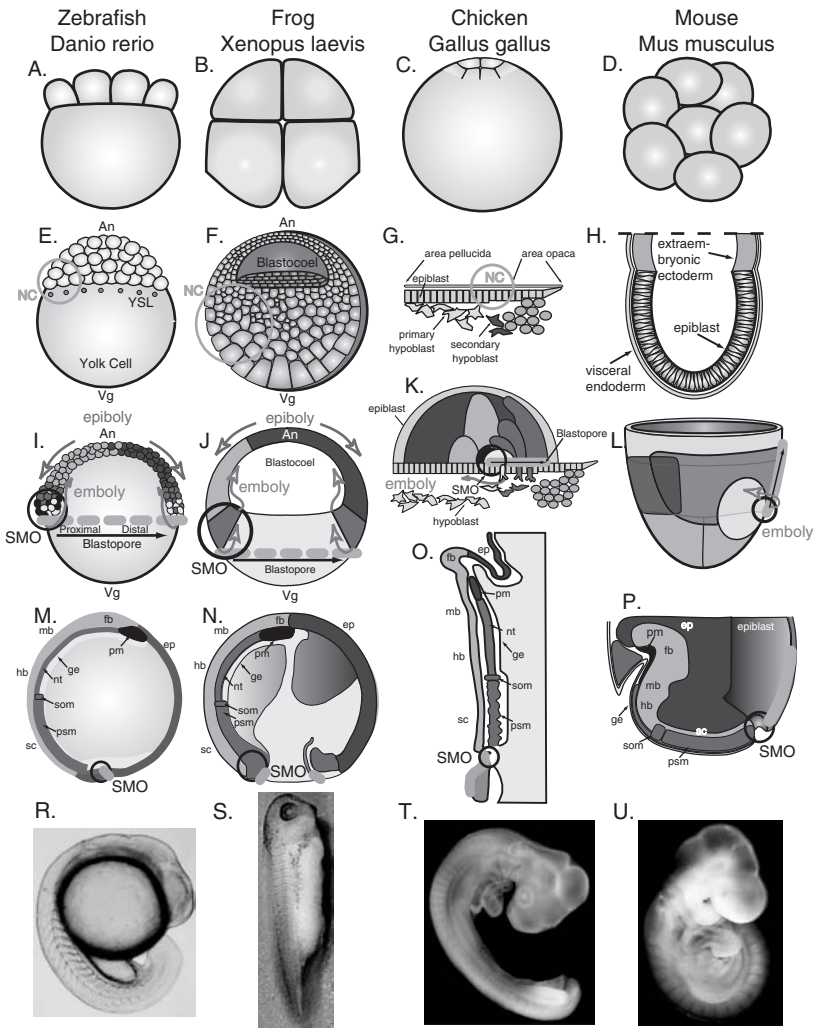

FIGURE 17.1 A comparison of the cleavage, blastula, and gastrula stages of four vertebrate model organisms: zebrafish, frog (*Xenopus laevis*), chick, and mouse. Developmental stages of zebrafish (**A, E, I, M, R**), frog (**B, F, J, N, S**), chick (**C, G, K, O, T**), and mouse (**D, H, L, P, U**). Cleavage, 8-cell stages (**A** through **D**). Note an incomplete cleavage in zebrafish (**A**) and chick (**C**), complete cleavage with different sizes of blastomeres in frog (**B**), and uniformly sized blastomeres in the mouse (**D**). Early blastula (**E** through **H**), late blastula to early gastrula (**I** through **L**), late gastrula (**M** through **P**), and pharyngula (**R** through **U**). The position of the Nieuwkoop center (NC) and its equivalents is shown in the cleavage stages, and the position of the Spemann–Mangold organizer region (SMO) is shown in the early and late gastrula stages. The gastrulation movements of epiboly and emboly are illustrated during the early gastrula stages (**I** through **L**). *Light gray,* Cytoplasm; *beige,* yolk, *dark gray;* epiblast region of amniote embryos; *red,* mesoderm and its precursors; *dark red,* prechordal mesendoderm; *yellow,* definitive endoderm and its precursors; *dark blue,* epidermis; *lighter blue,* neuroectoderm, *green, brown, and violet,* various extraembryonic tissues; *orange,* blastopore. *ep,* Epidermis; *fb,* forebrain; *mb,* midbrain; *hb,* hindbrain; *sc,* spinal cord; *nt,* notochord; *pm,* prechordal mesendoderm; *som,* somite; *psm,* presomitic mesoderm; *ge,* gut endoderm. (From Solnica-Krezel [2005], with permission. (See color insert.)

to address how gastrulation movements are orchestrated with patterning and cell fate specification events. Thus, with the use of different model systems, we are beginning to break down gastrulation into its many individual components. Although still far from complete, the emerging view of vertebrate gastrulation recognizes the conservation among species with regard to patterns of cell movement (Figure 17.2), gastrulation cell behaviors, and underlying molecular mechanisms (Solnica-Krezel, 2005).

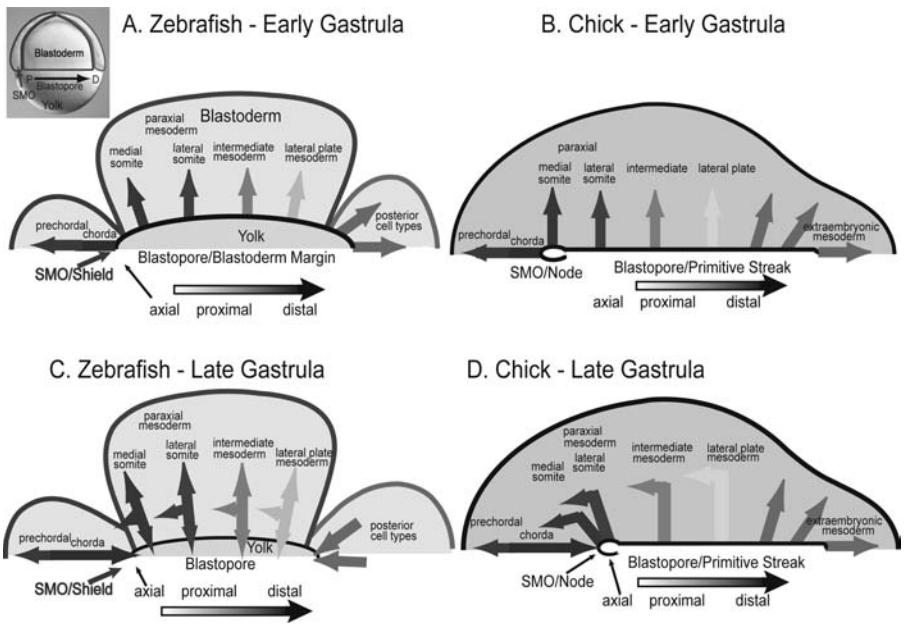

FIGURE 17.2 Conserved patterns of cell movements during vertebrate gastrulation. Patterns of cell movements during early (**A, B**) and late (**C, D**) stages of vertebrate gastrulation, with zebrafish (**A, C**) and chick (**B, D**) used as examples. **A,** Inset shows early zebrafish gastrula in a lateral view with animal to the top, vegetal to the bottom, and dorsal to the left. Blastopore is located at the blastoderm margin, with the Spemann–Mangold organizer taking its axial position. The arrow shows the proximal-to-distal axis of the blastopore. The schematic shows an imaginary flattened zebrafish embryo with the yolk cell below the blastopore or blastoderm margin. The blastoderm was flattened by dividing it into three regions (axial, proximodistal, and distalmost, as illustrated by the color lines on the figure of the live embryo). **B,** A schematic of chick gastrula depicted at stage 4+, when the primitive streak (blastopore) is most elongated. Only one half of the embryo on one side of the primitive streak is shown. Several similarities are apparent between **A** and **B.** Distinct types of mesoderm become internalized in similar regions of the blastopore: prechordal and chordal mesoderm move through the axial blastopore region and precursors of somites move via its proximal regions, whereas intermediate and lateral plate mesoderm move through successively distal regions. In the chick, the most distal aspect of the blastopore provides a conduit for the internalization of extraembryonic tissues. Alternatively, in zebrafish, the precursors of the most posterior (tail) somites, the intermediate and lateral plate mesoderm, move via the distal blastopore. Significantly, after internalization, all types of mesoderm move away from the blastopore. **C** and **D,** During the later stages of gastrulation, the blastopore becomes smaller. In fish, this is the result of epiboly movement; during chick gastrula, it is the result of the regression of primitive streak. Blastopore shrinking is linked with the extension of axial mesoderm. Streams of mesodermal cells that were internalized via proximal and proximodistal regions of the blastopore now move dorsally toward the extending midline tissues. Whereas, in the distal region of zebrafish blastopore, the prospective posterior mesoderm cells move back toward the blastopore to form tail bud (**C**), in the chick, there is a continued movement of extraembryonic mesoderm away from the blastopore (**D**). (Modified from Solnica-Krezel, 2005.)

III. EXPERIMENTAL APPROACHES TO STUDY VERTEBRATE GASTRULATION

Recent progress in our understanding of vertebrate gastrulation is in large part the result of the emergence of novel technologies that afford monitoring cell movements and cell behaviors *in vivo* at cellular and even subcellular resolutions. Moreover, molecular genetics, proteomics, and genomic methodologies are changing the landscape of developmental biology as well, affording mechanistic studies (Link et al., 2006; Wessely et al., 2004). Finally, classical genetic approaches, including forward genetic screens for mutations affecting gastrulation, have been applied to vertebrates, including zebrafish (Driever et al., 1996; Haffter et al., 1996), mouse (Kasarskis et al., 1998), and, more recently, frog (*Xenopus tropicalis;* Grammer et al., 2005). These screens have allowed us to identify and study genes with essential functions in gastrulation.

A. Embryology

Until recently, the direct and extended observation of embryos was limited to vertebrates that develop externally, as many fish and amphibian embryos do. In the absence of observational studies, domains of coordinated movement can be deduced on the basis of fate maps and changing patterns of gene expression (of course, the predictions need verification by observation). Additionally, movements can be inferred from the changing morphology revealed through light and electron microscopy (Lawson and Schoenwolf, 2001). Recently, conditions have been developed that support the normal development of mouse or chick embryos on the microscope stage, allowing for time-lapse analysis (Chapman et al., 2001; Passamaneck et al., 2006; Yang et al., 2002).

B. Imaging

Observation studies usually involve time-lapse recording to analyze the cell behaviors that underlie tissue morphogenesis. If the embryo is transparent, simple contrasting optics, such as differential interference contrast, may allow individual cells to be followed as has been demonstrated for teleost gastrulae (Trinkaus et al., 1992). In *Xenopus laevis,* as a result of the presence of opaque yolk granules in all cells, time-lapse observations were previously limited to tracking cells at the surface of intact embryos or explanted and exposed tissues. New technologies, such as surface imaging microscopy, provide three-dimensional resolution of cellular details in the optically opaque *X. laevis* embryo. In surface imaging microscopy, a computer-controlled microtome and an optical imaging system are used reiteratively to image and then remove the surface of the fixed embryo. The optical sections are subsequently used to reconstruct embryonic architecture in three dimensions (Ewald et al., 2002).

Cells can be labeled by the injection of fluorescent dyes or by the photoactivation of injected caged dyes to follow cell movement and/or cell fate (Kozlowski et al., 1997; Sepich and Solnica-Krezel, 2005). Organelles can be labeled with fluorescent fusion proteins targeted to specific cellular structure (e.g., histone–green fluorescent protein (GFP) labels the nucleus). Embryos may carry transgenes that encode fluorescent molecules under the control of tissue-specific promoters. This can allow tissues to be marked or inductive events to be monitored as gene expression is induced (Kimura-Yoshida et al., 2005; Passamaneck et al., 2006).

Several types of confocal microscopy systems have been developed to take fine optical slices of fluorescent samples. All of this optical information can be analyzed using a variety of software, both commercial and open-source freeware (e.g., Image J and Object Image).

C. Computational Modelling

Computational modeling has a long history in developmental biology (Meinhardt, 1978). As we will discuss later, many cellular behaviors occur during gastrulation. How do we assess the quantitative contribution of a single behavior to the body plan when the loss of a gene function frequently disturbs several aspects of morphogenesis? Modeling provides an efficient means of narrowing the range of hypotheses to be tested, because it easily reveals when a cell behavior is insufficient to drive the outcome. It allows us to eliminate one observed behavior at a time and to determine its contribution to the overall process. Finally, modeling can predict unanticipated or synergistic effects of genetic and experimental manipulation (Lewis, 2003; Longo et al., 2004).

D. Molecular Genetics

Molecules entered the field of gastrulation along with the first subtractive and expression cloning strategies. It became possible to clone genes with enriched expression in certain gastrula regions or having pronounced effects on patterning and/or gastrulation movements when misexpressed (Niehrs et al., 2001; Smith and Harland, 1992; Yamanaka et al., 1998). In addition, systematic *in situ* hybridization screens uncovered several genes with intriguing expression patterns during gastrulation, and they also delineated several synexpression groups, such as those comprised by the fibroblast growth factor (FGF) pathway components (Fürthauer et al., 2002; Niehrs and Pollet, 1999). These strategies are being currently replaced by microarray approaches, which provide high-throughput means to identify genes expressed at specific times during gastrulation, in specific gastrula regions, or that are expressed dynamically in response to experimental or genetic manipulations (Wessely et al., 2004).

E. Reverse Genetics

Several methods are now available in many model systems to query the function of the candidate gastrulation genes identified by the gene discovery strategies outlined above. The mouse is the most sophisticated vertebrate in this regard, with elegant methods available for targeted gene manipulation in the whole animal or at specific stages of development and in specific tissues (Capecchi, 2005). Other vertebrate model systems (including frog, fish, and chick) use various gene interference methods. These include RNAi, where antisense RNAs target cognate transcripts for degradation, and antisense morpholino oligonucleotides, which interfere with either the translation or splicing of targeted transcripts (Heasman et al., 2000; Nasevicius and Ekker, 2000). Many proteins also lend themselves to a dominant negative strategy whereby a mutant protein not only lacks normal activity but also inhibits the normal activity of the endogenous protein (Amaya et al., 1991). All of these methods require the careful assessment of their specificity to determine whether the observed phenotypic consequences are the result of the altered activity of only one or possibly other genes. The degree to which a gene function is impaired by these tools is also an important experimental consideration.

F. Forward Genetics

Genetic screens for mutations that disrupt specific developmental processes have been very effective for determining the mechanisms of development in invertebrate animals like flies and worms (reviewed in Anderson and Ingham, 2003). Such an unbiased genetic approach has been applied to gastrulation in zebrafish (Hammerschmidt et al., 1996; Kane et al., 1996; Solnica-Krezel et al., 1996) and, more recently, in the mouse (Kasarskis et al., 1998; Zohn et al., 2005). Genetic screens for recessive zygotic mutations in both systems took advantage of the high efficiency with which the alkylating agent *N*-ethyl-*N*-nitrosourea can mutagenize the germline. In the zebrafish genetic screens, mutagenized males (F0) are crossed with wild-type females to produce F1 progeny, each of which is heterozygous for a unique set of mutations (Figure 17.3). Subsequently, F1 animals are crossed with wild-type fish, or two F1 animals are mated to combine mutations in F2 lines, in which 50% of animals are heterozygous for mutations inherited from F1 parents. Mutations in the F2 families are identified by mating F2 siblings and screening the resulting progeny at several stages of embryonic development (days 1, 2, 3, and 5 after fertilization; Hafter et al., 1996; Driever et al., 1996). As expected from mendelian segregation of the induced recessive mutations, 25% of F3 embryos in 25% of such F2 sibling crosses would manifest a given mutant phenotype. A number of the features of the zebrafish model facilitate such screens; zebrafish fertilization and entire embryonic development occur externally, and the resulting embryos and their chorions are transparent, thus affording the direct examination of the morphology and function of not only the external but also the internal organs. In particular, many mutations affecting gastrulation movements have been identified by virtue of the abnormal morphology of mutant embryos (Hammerschmidt et al., 1996; Kane et al., 1996; Solnica-Krezel et al., 1996).

Genetic screens in the mouse have used a similar breeding scheme, except that F2 females are back-crossed to their fathers. As a result of intrauterine development in the mouse, the F3 embryos are analyzed only once, at 9.5 days postcoitum, when embryos that have defects during early development would manifest abnormal morphology but would not have been entirely resorbed (Kasarskis et al., 1998). Although it is labor intensive, this genome-wide genetic screen in the mouse is identifying new regulators of gastrulation (Zohn et al., 2006). Likely, forward genetic screens in zebrafish, mouse, and frog *(X. tropicalis)* will continue to parallel and complement the reverse genetic approaches discussed previously.

IV. EPIBOLY

During the course of fish and frog gastrulation, the prospective superficial ectoderm must spread to cover the internalized mesendoderm and, in the fish, also the yolk cell (see Figure 1, I and J). Similarly, in amniote embryos, the initially small blastodisc increases in surface area. The process of tissue expansion, which is often accompanied by thinning, is known as *epiboly* (Figure 17.4). The best-characterized examples of epiboly are the epithelial thinning of the blastocele roof in *X. laevis* blastulae and the thinning and spreading of the blastoderm in zebrafish (Keller, 1980; Warga and Kimmel, 1990). The *X. laevis*

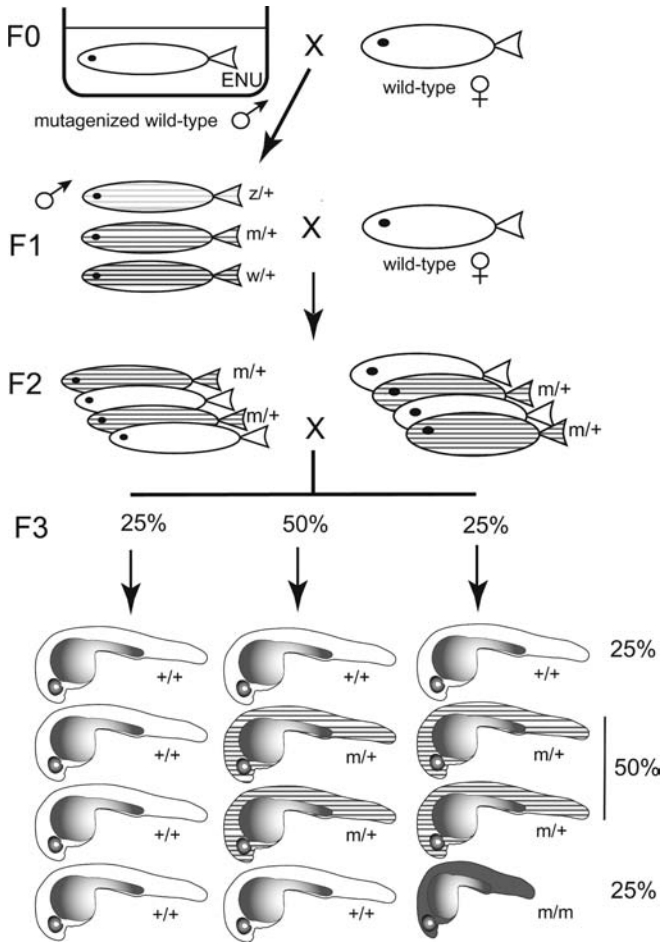

FIGURE 17.3 **Strategies for classic genome-wide genetic screens for zygotic recessive mutations affecting gastrulation in zebrafish and mouse.** An F2 screening strategy in zebrafish after the mutagenesis of premeiotic germ cells (spermatogonia). After the mutagenesis of adult F0 males by incubation several times in *N*-ethyl-*N*-nitrosourea solution, adult sperm cells contain mutations in different genes. The mutagenized F0 males are crossed with wild-type females to produce thousands of phenotypically normal F1 progeny, each of which is heterozygous for mutations in a unique set of genes. Two F1 fish are intercrossed to produce an F2 family in which 50% of phenotypically normal F2 fish are heterozygous for any particular induced mutation inherited from either F1 parent. Mutations are uncovered by mating F2 siblings and the microscopic observation of the resulting F3 progeny from each F2 cross. Consistent with Mendelian segregation is the fact that each mutation is manifested in one quarter of the progeny in one quarter of matings. The screening of six crosses from an F2 family affords an 82% chance of identifying any given mutation. The same mutation can appear in more than one cross, and different crosses can show mutations in different genes inherited from the same or the other F1 parent. (Based on Mullins et al., 1994, and Solnica-Krezel et al., 1994.)

blastocele roof is initially composed of three to four cell layers. The most superficial layer has epithelial character, whereas the deeper cells are more mesenchymal. During the course of epiboly, these deeper cells engage in radial intercalation, whereby cells interdigitate radially among their more superficial neighbors, which drives both the thinning and the expansion of this tissue (Figure 17.4, B). Simultaneously, the surface layer expands as its cells flatten (Figure 17.4, C). Similarly, in the zebrafish gastrula, more deeply located cells

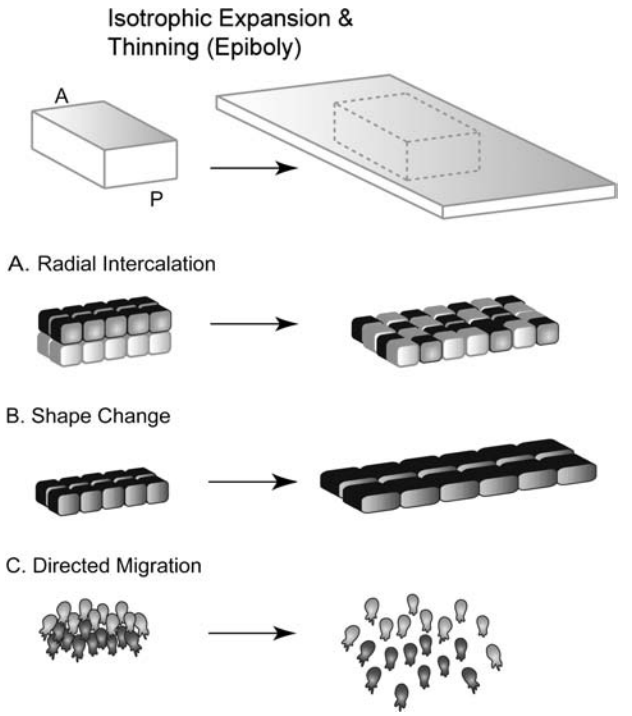

FIGURE 17.4 **Epiboly leads to the isotropic expansion of tissue, often accompanied by its thinning.** Three cell behaviors that can generate spreading and thinning are detailed. **A,** Radial cell intercalation entails the intrusion of cells from one layer into an adjacent layer, resulting in the thinning and surface expansion of tissue. **B,** Changes in cell shape, here flattening and spreading either an epithelium or a mesenchymal array of cells. **C,** Dispersive, directed migration of tightly packed mesenchymal cells leads to their spreading and thus the surface expansion of tissue.

intercalate radially among their more superficial neighbors, and they become flattened as they enter the superficial layer. These cells do not reenter the deeper layers (Kane et al., 2005).

In both systems, intercellular adhesion as well as the interaction of cells with the extracellular matrix is essential for epiboly. Studies in the frog showed that the thinning of the blastocele roof via radial intercalation is dependent on fibronectin, which is secreted and assembled into extracellular fibrils by the deeper cells (Longo et al., 2004). Current studies in zebrafish provide genetic evidence that E-cadherin, a homophilic calcium-dependent cell adhesion molecule, is necessary for this process (Kane et al., 2005; Shimizu et al., 2005). During epiboly, E-cadherin RNA forms a gradient with low levels in the deep cell layers and increasing levels toward the superficial cell layer; this gradient could account for a polarized radial intercalation of cells from the deeper to the superficial layers (Kane et al., 2005). In *half baked* (*hab*, *E-cadherin*) mutants, although cells intercalate into the superficial layer, they often fail to maintain this position and thus fall back into the deeper layer. Moreover, when they are transplanted to wild-type embryos, mutant cells that intercalated into the superficial layer fail to acquire the flattened cell morphology that is typical of the superficial layer. Another report argued for decreased rates of radial intercalation in the dorsal organizer region of zebrafish embryos in which E-cadherin levels were reduced with antisense morpholino oligonucleotides (Montero et al., 2005). These studies

demonstrate that the radial intercalation of deep cells into the superficial layer and the flattening of the cells in the superficial layer drive zebrafish epiboly and that E-cadherin plays a crucial role in these cell behaviors (Kane et al., 2005). Epiboly may require both optimal level and dynamic regulation of cell adhesion molecules; this notion is suggested by the observation that radial intercalation and epiboly in the frog *X. laevis* were impaired upon N-cadherin overexpression (Longo et al., 2004).

V. INTERNALIZATION

The movement of prospective mesoderm and endoderm from the surface into the gastrula interior is the gastrulation movement conserved from invertebrate through vertebrate animals (Leptin, 2005). This movement creates the triploblastic body plan, with the mesoderm becoming the intermediate layer sandwiched between the most internal endoderm and the superficial ectoderm. Mesodermal and endodermal precursors internalize via a portal known as the *blastopore* (see Figure 17.1, I through L). In *X. laevis,* this opening forms just vegetally to the SMO region as the so-called dorsal blastopore lip. Subsequently, the dorsal lip expands mediolaterally to eventually fuse on the ventral side of the gastrula (Keller and Shook, 2004; Shih and Keller, 1994). In zebrafish, the margin of the blastodermal cup adjacent to the yolk cell serves the role of the blastopore (Warga and Kimmel, 1990). In amniotes, the blastopore is known as the primitive streak, which forms as a small opening in the midline of the caudal aspect of the epiblast and from there extends rostrally (see Figure 17.2; Stern, 2004; Tam and Gad, 2004).

Whereas internalization is evolutionarily conserved, there are similarities and differences in the patterns of internalization of different types of mesoderm through the blastopores of diverse vertebrate gastrulae (see Figure 17.2). In fish and amphibians, the prospective axial and the dorsoanterior mesendoderm internalize via the blastopore regions proximal to the SMO (Melby et al., 1996; Shih and Fraser, 1995; Shih and Keller, 1994). By contrast, in amniote gastrulae, internalization is initiated in the blastopore region distal to the node, and it involves extraembryonic and prospective posterior mesoderm (Vakaet, 1970). Despite this difference in the location of the initial internalization movements, the spatial order in which distinct types of mesoderm internalize via a blastopore relative to the SMO are markedly similar. In all vertebrates, axial mesoderm precursors comprising prechordal and chordamesoderm become internalized in the axial blastopore, where the SMO resides. Paraxial mesoderm, including head and somitic mesoderm precursors, moves inside via the blastopore region proximal to the SMO, whereas the intermediate and then lateral plate mesoderm precursors move via regions of the blastopore progressively distal to the organizer (Schoenwolf et al., 1992; Stern, 2004). Finally, the posterior mesendodermal precursors in zebrafish (Kanki and Ho, 1997; Myers et al., 2002a) and amniote extraembryonic mesoderm internalize via the distal-most regions of the blastopore (Lawson, 1991; Psychoyos and Stern, 1996).

The cellular behaviors responsible for the process of internalization also vary among vertebrates (Figure 17.5, A through C). In the best-studied process of involution in *X. laevis,* mesendodermal precursors form a sheet that bends and turns inward and back on its inner surface in the blastopore region

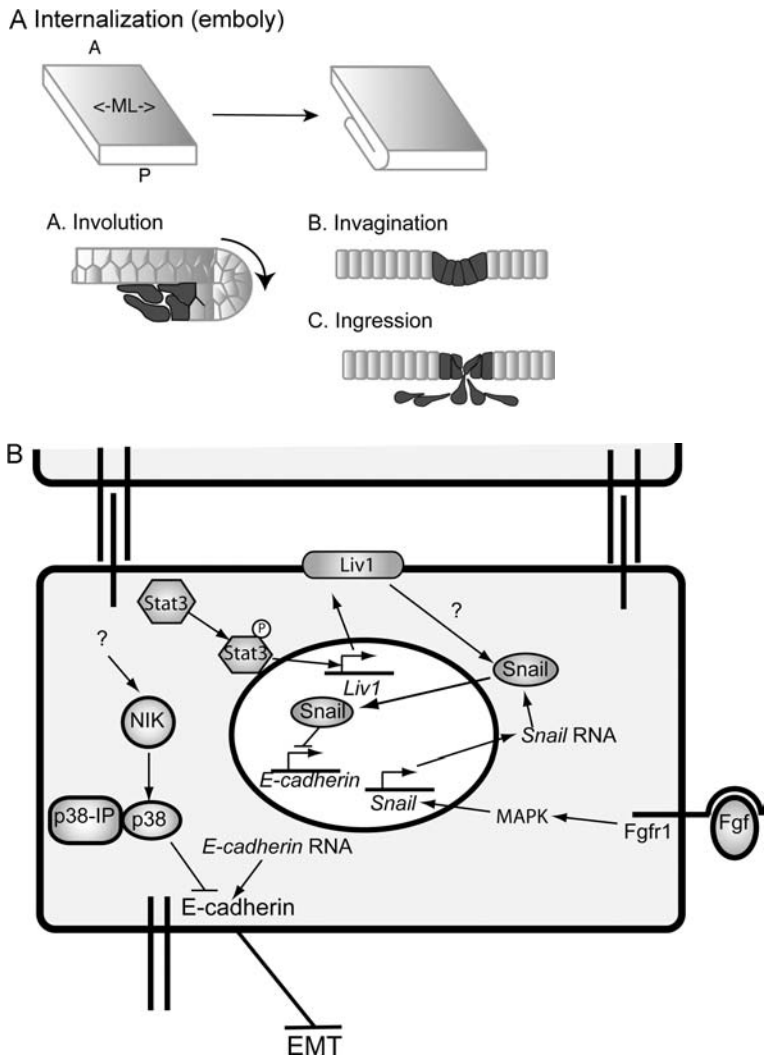

FIGURE 17.5 **Emboly or internalization entails movements of mesodermal and endodermal precursors from the blastula surface beneath the prospective ectodermal layer. A,** Involution entails the rolling of a cell sheet over an edge (blastopore) and frequently upon itself. Upon involution, cells at the leading edge can undergo various degrees of epithelial-to-mesenchymal transition (EMT) and move upon the overlying sheet as a substratum. **B,** Invagination, or the formation of a groove (blastopore) in a sheet of tissue, occurs via cell shape changes, such as apical constriction. **C,** Invagination is often followed by ingression, whereby cells in the groove undergo EMT, break away from the cellular sheet, and move freely beneath the surface layer. **D,** Downregulation of E-cadherin expression and activity is a hallmark of EMT and internalization during gastrulation in many vertebrates. Transcriptional repression of E-cadherin gene is accomplished by the transcription factor Snail, the expression of which is tightly regulated by several pathways at transcriptional, protein stability and localization levels.

such that, upon internalization, its leading edge faces animally (Keller and Davidson, 2004). Thus, the process of involution entails the movement and bending of an epithelial-like sheet. By contrast, during amniote ingression, prospective mesodermal precursors residing in the epithelial tissue at the blastopore individually break away from the epithelial sheet to move through the blastopore. Finally, in the zebrafish, mesendodermal precursors gathered as a

coherent tissue at blastoderm margin move via the blastopore in an orderly fashion as groups of cells in a process known as a *synchronized ingression* (Adams and Kimmel, 2004).

Thus, both in amniotes and in fish, the process of internalization is associated with an alteration of tissue architecture: a coherent and less motile tissue breaks into groups or individual motile cells or acquires a less cohesive organization. Alternative, in frogs, involution involves the bending of cellular sheets, and the leading edge of the internalized mesodermal sheets breaks into migrating individual cells (discussed later). The phenotypic transition from a coherent sheet with apical–basal polarity to a less coherent, more individually motile group of cells without clear apical–basal polarity is known as the EMT (Leptin, 2005; Shook and Keller, 2003).

Genetic studies of different vertebrates have demonstrated that the process of internalization is absolutely dependent on secreted molecules that activate the Nodal, FGF, and canonical Wnt signaling pathways, which are all required for the specification of the mesodermal cell fate. Nodal signaling is also essential for endoderm specification (Schier, 2004). Zebrafish and mouse embryos in which Nodal signaling has been attenuated by the inactivation of the components of this pathway or frog embryos in which Nodal signaling has been inhibited with a specific antagonist fail to form axial and paraxial mesoderm, whereas posterior mesodermal fates are relatively intact (Agius et al., 2000; Conlon et al., 1994; Feldman et al., 1998; Gritsman et al., 1999). Interference with FGF signaling during gastrulation using mutational or dominant negative approaches results in a deficiency of paraxial and posterior mesodermal fates without significantly affecting axial and anterior mesoderm (Amaya et al., 1991; Yamaguchi et al., 1994). Similarly, primitive streak and posterior mesoderm deficits have been reported for fish and mouse embryos that are defective with regard to Wnt3 signaling (Liu et al., 1999). In all of the above scenarios, mesoderm-specific markers such as T-box–related transcription factors are not expressed or expressed at reduced levels, and excess neural tissues form at the expense of mesoderm. Moreover, the process of internalization is inhibited, and mutant embryos display ectopic cells accumulating at the blastopore (Ciruna and Rossant, 2001; Ciruna et al., 1997).

Although detailed molecular genetic mechanisms regulating internalization are not understood, some important players have been identified. One common molecular change associated with and required for the EMT during internalization is the downregulation of E-cadherin (Figure 17.5, D). This cell adhesion molecule is prominent on the cell membranes of epithelial cells, but it is present at significantly lower levels in internalizing mesenchymal cells, and this allows for the breakdown of the epithelial organization. The downregulation of E-cadherin is achieved largely through the repression of its transcription by the transcription factor Snail, which provides a link between the Nodal and FGF pathways and EMT (Barrallo-Gimeno and Nieto, 2005; Carver et al., 2001). Indeed, transcription of the *snail* gene is dependent on both Nodal and FGF signaling in mouse and zebrafish (Ciruna and Rossant, 2001; Gritsman et al., 1999). Recent genetic studies in the mouse revealed that the downregulation of E-cadherin also requires P38 and P38 interacting proteins, which act downstream of Nck-interacting kinase (NIK) in a pathway that is parallel to *snail* transcription (Zohn et al., 2006). Yet another pathway regulating Snail nuclear localization and activity has been identified in zebrafish. Stat3 signaling in the internalizing prechordal mesoderm cells activates the

expression of Liv1, a zebrafish homolog of a breast-cancer–associated zinc transporter that in turn promotes the nuclear localization of Snail transcription factor, a negative regulator of E-cadherin expression (Yamashita et al., 2004). Therefore, a number of different pathways cooperate in the downregulation of E-cadherin levels to allow for EMT and internalization (Figure 17.5, D). Whether all of the above pathways are employed during internalization in different vertebrate gastrulae remains to be determined.

As the mesoderm internalizes, it is important that it remain separate and that it not merge into the ectoderm. The inability to maintain separation is associated with reduced or failed gastrulation (Montero et al., 2005; Wacker et al., 2000). Wnt/Ca signaling, cadherin, and protocadherin have been implicated in this process (Medina et al., 2004; Winklbauer et al., 2001).

VI. CONVERGENCE AND EXTENSION MOVEMENTS

After the internalization of mesendodermal precursors via different regions of the blastopore, all three germ layers undergo C&E to form a rostrocaudally elongated and mediolaterally narrowed embryo. C&E movements are driven by a combination of cell behaviors that vary according to their position in the gastrula and their stage of development (Figure 17.6, A through E). Here, we will consider the C&E movements of two different mesodermal cell populations: the axial mesoderm that is internalized via the SMO region of the blastopore and the prospective lateral plate mesoderm that internalizes via the proximal–distal blastopore region (see Figure 17.2). After internalization, both of these mesodermal cell populations move away from the blastopore (fish and frogs) or the primitive streak (mice and chicks). This is the first movement that contributes to the extension of mesodermal tissues along the rostrocaudal (anteroposterior) embryonic axis (see Figure 17.6, A). A second cell behavior that has been reported in fish contributes in a subtle way to the extension of the axis throughout gastrulation in both ectoderm and mesoderm. In the dorsal half of the embryo, the plane of cell division is oriented so that daughter cells align with the anterior–posterior axis and thus add to axis extension (see Figure 17.6, E; Gong et al., 2004).

A. Axial Mesoderm

Axial mesoderm gives rise to the midline mesodermal tissues, the prechordal plate, and the notochord, and it internalizes through the blastopore, where the SMO resides (the embryonic shield in fish, the dorsal blastopore lip in frog, and the node of avian gastrulae; see Figure 17.1, I through L). The anteriormost are prechordal mesoderm cells, which contribute to the head. They migrate anteriorly as a loose cohort with anteriorly oriented protrusions (Lawson and Schoenwolf, 2001; Ulrich et al., 2003; Winklbauer and Nagel, 1991).

The precursors of the notochord follow the head mesoderm. They move as a cohesive tissue in all vertebrates (see Figure 17.2). This tissue, the chordamesoderm, undergoes a dramatic cellular rearrangement (described in *X. laevis* in Keller et al., 2003) whereby bipolar cells elongate mediolaterally and push between neighboring cells to displace them anteriorly or posteriorly (see Figure 17.6, A). Such mediolateral intercalation behavior (MIB) results in simultaneous C&E, and it is called *convergent extension* to reflect its

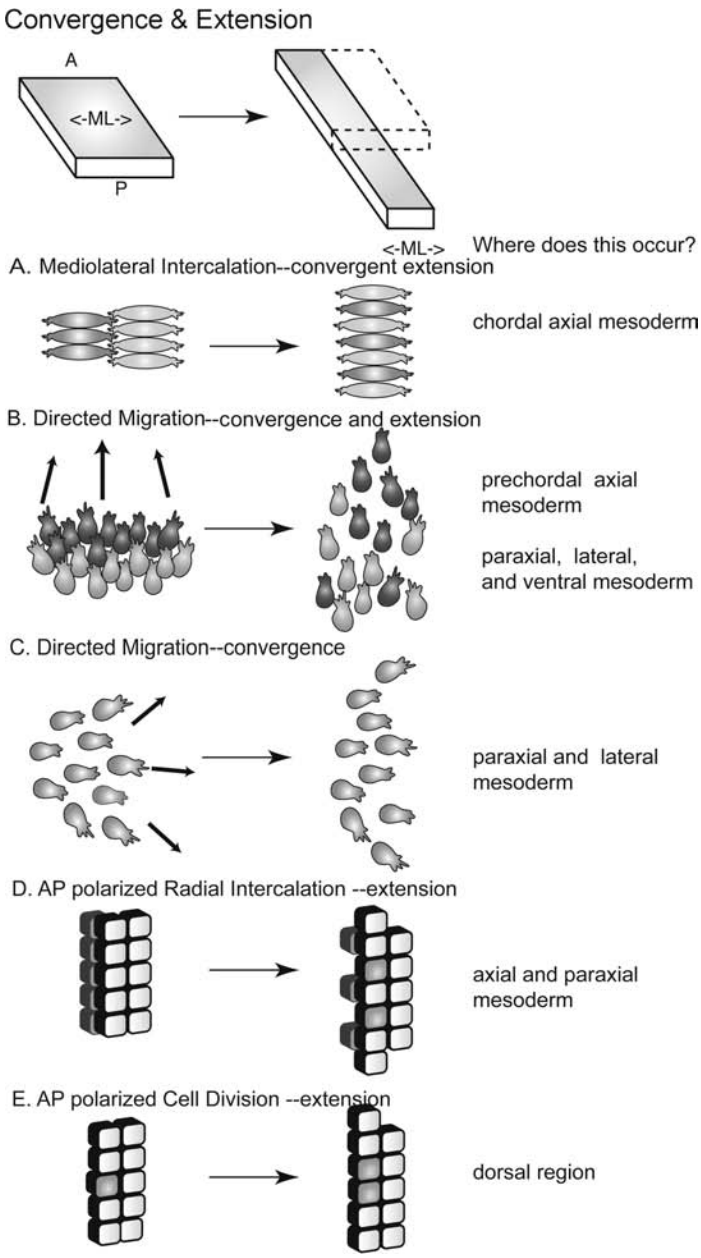

FIGURE 17.6 **Convergence movements narrow embryonic tissues mediolaterally, whereas extension movements elongate them from head to tail.** This morphogenetic change can be achieved by a variety of cell behaviors, including the following: **A,** Intercalation of mediolaterally elongated cells between their medial and lateral neighbors results in simultaneous the convergence and extension of tissue. **B,** Directed migration away from the blastopore and anteriorly extends all nonaxial mesoderm and assists with the anterior migration of the prechordal mesoderm in frog and fish embryos. **C,** Directed migration toward the dorsal midline. Some cells move exclusively dorsally, some cells show dorsal–animal bias, and some cells show dorsal–vegetal bias. This can also lead to the convergence and extension of tissue as observed for lateral mesoderm in zebrafish gastrula. **D,** Polarized radial intercalation of cells between anterior and posterior neighbors in the adjacent layer in axial and paraxial mesoderm extends tissues. **E,** Cell division aligned with the anterior–posterior axis contributes to extension.

linked nature. At the lateral edges of the notochord, a boundary forms that separates the notochord from the paraxial mesoderm. Protrusions that contact the lateral boundary cease activity. This boundary is believed to be essential to turn MIB into tissue narrowing and extension by limiting protrusions and movement to the medial direction (Keller et al., 2003). The movement behavior of the notochord precursors in zebrafish conforms well to the predictions of the mediolateral intercalation model (Glickman et al., 2003). The mutant *notail* (*ntl/Xbra/T-gene*) lacks MIB and convergent extension; however, some extension can still occur as cells spread apart in a rostrocaudal direction without convergence, possibly through animalward migration or epiboly driven by radial intercalation (see Figure 17.6, *B*; Glickman et al., 2003). In *X. laevis,* paraxial or somitic mesoderm also engages in mediolateral intercalation behavior (described later). The Wnt signaling pathway is required for the efficient narrowing and extension of the notochord and somites and the migration of the prechordal mesoderm cells (see Wnts later in this chapter).

The transition in axial mesoderm from cell migration in the prechordal plate rudiment to convergent extension in the notochord anlage correlates with the expression of *notail* (*ntl/Xbra/T-gene)*, a T-box transcription factor (Kwan and Kirschner, 2003; Schulte-Merker et al., 1994; Tada and Smith, 2000). *X. laevis* blastoderm cells can be induced to exhibit migration or MIB in culture. Blocking Xbra inhibits the MIB behavior, but it has little effect on migration. Conversely, excess Xbra partially inhibits migration, but it does not inhibit MIB or, consequently, convergent extension (Kwan and Kirschner, 2003). Likewise, in zebrafish *ntl* mutant gastrulae, the MIB of chordamesoderm cells is impaired, but other cell movement behaviors appear normal (Glickman et al., 2003). These results indicate that the expression of Xbra serves as a switch between distinct cell movement behaviors.

B. Lateral Mesoderm

The mesoderm that gives rise to the somites, kidneys, heart, and blood is distributed along the region between the notochord and the ventral gastrula midline. This mesoderm arises from proximodistal blastopore regions (see Figure 17.2), and it uses several different cell behaviors to converge and extend. These behaviors change during gastrulation, reflecting the change in tissue organization that occurs when mesoderm cells internalize and then migrate as individual cells. As the cells accumulate dorsally, they again form a cohesive tissue that thickens, converges, and extends via cell migration and intercalation. *X. laevis* is the exception to this strategy; its mesoderm remains in a sheet during early gastrulation, engaging in mediolateral intercalation behavior as described previously for axial mesoderm (see Figure 17.6, A). Like axial mesoderm, newly internalized lateral cells move away from the blastopore (see Figure 17.6, B). This movement extends the mesoderm anteroposteriorly in fish and frogs, whereas it widens the mesoderm mediolaterally in chicks and mice.

Convergence begins when paths of the migrating mesodermal cells turn toward the midline and the notochord anlage during gastrulation (see Figure 17.6, C; Sepich et al., 2005; Yang et al., 2002). In the teleost fish, these loose cells travel on meandering but dorsally oriented paths (Jessen et al., 2002; Trinkaus, 1998). Paths also fan out along the anterior–posterior axis

so that more rostral cells move toward the head as well as the midline (Sepich et al., 2005). In the chick, these mesodermal cells, when transplanted, move correctly for their new location, which suggests that they respond to local cues and potentially to chemotactic signals (see Fibroblast Growth Factors later in this chapter; Yang et al., 2002).

In the zebrafish, cell behaviors that contribute to C&E of the lateral mesoderm change again during late gastrulation. Lateral cells become closely packed and appear to form a sheet. Cells are elongated and aligned mediolaterally; they converge on straight direct paths as a cohort without mediolateral intercalation (Jessen et al., 2002). Approaching segmentation stages, C&E continues in the paraxial mesoderm of both fish and frog embryos using several new cell behaviors (Henry et al., 2000; Wilson et al., 1989). Cells extend the mesoderm through both mediolateral and radial intercalations. The radially oriented cell intercalations preferentially separate anterior–posterior neighbors, extending the mesoderm (Figure 17.6, D; C. Yin and LSK, unpublished), as hypothesized previously (Wilson et al., 1989). These late gastrulation cell behaviors depend on noncanonical Wnt signaling (see Wnt later in this chapter).

Although the variety of cell movements in C&E are shared among the vertebrates, the set of behaviors displayed during the gastrula of one species may vary from those of another species. In addition, the behavior demonstrated by a single population of lateral mesodermal cells changes at different stages of gastrulation, and the behaviors displayed by adjacent but distinct types of mesoderm may differ (e.g., head and chordamesoderm). This assortment of regionally and temporally controlled movements implies the complexity of signaling pathways that regulate C&E.

C. Molecular Mechanisms of Convergence and Extension

Cells have robust mechanisms to modulate adhesion and to drive the formation of the structures that underlie motility. Morphogenesis requires that these mechanisms be expressed locally, in a context-specific manner. In this section, we will focus on a few of the best-characterized molecules that regulate C&E. First, we will look at the molecules that are thought to provide directional cues: (1) FGFs acting as chemorepellents in the chick blastopore and as chemoattractants in the chick midline; and (2) platelet-derived growth factor (PDGF) in frogs and fish. We will also consider Stat3, a transcription factor that regulates movement in lateral and axial mesoderm. Next, we will discuss molecules that might not influence the direction of cell movement but rather the movement efficiency. In particular, we will focus on Wnt signaling, which is needed for several polarized cell behaviors in axial and lateral mesoderm. We will discuss its interactions with cell–cell adhesion (cadherin, protocadherin, and ephrin) and cell–matrix adhesion systems (fibronectin, syndecan, $\alpha 5\beta 1$ integrin). We will end with heterotrimeric G proteins that regulate several aspects of motility and with effectors of FGF-mediated motility.

1. FGFs as Chemorepellents and Chemoattractants Guiding Convergence and Extension Movements of the Internalized Mesoderm

The directional migration of the internalized mesodermal cells away from the blastopore and later toward the dorsal midline (see Figure 17.2) appears to depend on environmental cues. These cues might be a gradient of chemoattractant diffusing from a midline source or a gradient of cues supplied by the

local substratum (e.g., a gradient of adhesion). FGFs serve multiple duties in the regulation of gastrulation movements (Sivak and Amaya, 2004). Genetic studies in the mouse, which were described previously, revealed a role for FGF signaling in EMT during internalization. Work in the chick argues for an additional instructive role of FGFs in the movements of the internalized mesoderm away from the primitive streak and later for its convergence toward the midline (Yang et al., 2002). During chick gastrulation, FGF8 is expressed in the primitive streak (blastopore) consistent with a proposed role as a chemorepellent to drive mesodermal cells away from the blastopore. Accordingly, FGF8-soaked beads embedded in the chick gastrula can repel migrating mesodermal cells. In the opposite role, FGF4 is expressed at the anterior end of the primitive streak, in the forming notochord. As the notochord at the node extends, the blastopore is displaced and shortened (see Figure 17.2, *B* and *D*). Consistent with a role for FGF4 as a chemoattractant is the fact that mesodermal cells migrate toward the midline after the node regresses past them. Both the nascent notochord and FGF4-soaked beads can attract mesodermal cells when they are grafted onto the chick embryo (Yang et al., 2002). How FGF signals are translated into the directed cell migration behavior and whether or which FGFs might act as chemoattractants in other vertebrate gastrulae remain to be determined.

2. Platelet-Derived Growth Factor

PDGF is involved in the anterior migration of internalized cells in *X. laevis* and zebrafish. In the frog gastrula, the anterior migration and survival of mesodermal cells depend on the function of PDGFα receptors in the mesoderm and on its ligand, PDGF, which is secreted by the ectoderm. In the absence of PDGF, *X. laevis* cells continue to migrate, but without anterior orientation (Nagel et al., 2004; Van Stry et al., 2005). Although PDGF and its receptor are expressed ubiquitously in zebrafish gastrulae, interference with their signaling also impairs the anterior orientation of protrusions and cell migration (Montero et al., 2003). PDGF acts through phosphoinositide 3-kinase and its downstream effector protein kinase B. Protein kinase B accumulates in locations in which protrusions form and in the anterior direction (Montero et al., 2003). At this point, it is not clear if PDGF acts as a weak chemoattractant in some animals or as a factor that enhances mesoderm's ability to migrate directionally.

3. Stat3

Studies in zebrafish revealed that Stat3 is involved in two aspects of C&E movements (Yamashita et al., 2002). First, it is needed cell autonomously for prechordal mesoderm to migrate anteriorly, away from the blastopore. In a second role, Stat3 is required for prechordal mesoderm to provide an attracting influence on the convergence of lateral mesodermal cells. In zebrafish, ubiquitously expressed Stat3 is activated on the dorsal side of the blastula and later in the prechordal mesoderm (Oates et al., 1999; Yamashita et al., 2002). Notably, the dorsal activation of Stat3 occurs independently of germ layer induction and patterning, which reveals that patterning and morphogenetic events during gastrulation can be at least partially uncoupled. The loss of Stat3 function impairs both the convergence of lateral mesoderm and the extension of prechordal mesoderm. However, both defects can be suppressed

by restoring Stat3 expression in the axial mesoderm alone. By contrast, Stat3 function in the lateral mesoderm alone cannot restore lateral or dorsal cell migration. These results are interpreted to mean that Stat3 as a transcription factor turns on a set of genes in the prechordal mesoderm for cell-autonomous functions, like cell motility, and for nonautonomous functions, like the production of signals that attract lateral mesoderm (Yamashita et al., 2002). Whether Stat3 has similar functions during gastrulation in other vertebrates remains to be determined.

What downstream pathways does Stat3 regulate during C&E movements of the axial mesoderm? Liv1, a zinc transporter protein, appears to be a downstream target of Stat3 that is required in prechordal mesoderm cells for their anterior migration, but it has no effect on the convergence of lateral mesoderm (Yamashita et al., 2004). Liv1 is hypothesized to support prechordal mesoderm cell migration by reducing cell adhesion through Snail1, a transcription factor associated with EMT. Snail1 represses the transcription of E-cadherin. However, other work indicates an increase in E-cadherin adhesion when cells internalize (Montero et al., 2003). As discussed previously, it is not clear whether the main role of the Liv1–Snail–E-cadherin pathway is to promote EMT during the internalization process, to promote the anterior migration of prechordal mesoderm upon internalization, or both.

Stat3 has a cell nonautonomous role in the prechordal mesoderm to attract lateral mesoderm (Yamashita et al., 2004). Via an unknown pathway, Stat3 activates the small GTPase Rho in lateral cells, and it causes cells to elongate and mediolaterally align (Miyagi et al., 2004). Wnt and Stat3 signaling each contribute to the mediolaterally aligned and elongated cell phenotype of lateral mesoderm at late gastrulation. Cell elongation requires Rho GTPase activity, whereas mediolateral alignment appears to require a community of properly behaving cells (Marlow et al., 2002). Stimulating the planar cell polarity (PCP)/Wnt pathway can restore elongation in Stat3-depleted lateral cells. The addition of a Stat3-directed signal from the prechordal plate aligns these Wnt-elongated lateral cells; this is consistent with its proposed role as the regulator of a directional signal (Miyagi et al., 2004).

4. Wnts

Wnt signaling acts through the canonical Wnt pathway to regulate gene transcription and cell fate. Wnts also stimulate other noncanonical pathways that share some downstream components with the canonical pathway (Veeman et al., 2003; Wallingford et al., 2002). The PCP/Wnt signaling pathway influences cell polarity. In *Drosophila melanogaster,* the PCP pathway coordinates the growth direction of wing hairs in an epithelium so that they point to the distal tip of the wing (Klein and Mlodzik, 2005). In the vertebrate gastrulae, individual cells can exhibit monopolar or bipolar protrusions within the plane of the cell layer. PCP acts to organize these separate polarities over a tissue. In vertebrates, this pathway is stimulated by Wnt ligands, and it is involved in C&E. An important difference in PCP/Wnt signaling between *D. melanogaster* wing polarity and vertebrate gastrulation movements is the stability of neighbor cell relationships. In an epithelium, like in the wing primordium, neighbors remain in contact for many hours. However, during C&E movements (particularly those involving intercalating mesenchymal cells), neighbors can change frequently.

Impaired PCP/Wnt signaling during gastrulation results in embryos with reduced C&E that are shorter rostrocaudally and broader mediolaterally. PCP/Wnt function is required autonomously in both axial and lateral mesoderm for convergence. Wild-type PCP/Wnt function in the axial mesoderm cannot rescue cell movements in PCP/Wnt-impaired lateral mesoderm (Heisenberg et al., 2000). Like Stat3, PCP/Wnt signaling regulates C&E movements without influencing patterning during gastrulation (Heisenberg et al., 2000; Klein and Mlodzik, 2005). Current studies do not support the notion that PCP/Wnt signaling provides directional cues. Rather, PCP/Wnt is required for the effective movement of cells toward other cues, such as FGFs and the targets of Stat3 discussed previously (Jessen et al., 2002).

PCP/Wnt signaling regulates a multitude of cellular properties and behaviors to promote directional and efficient C&E movements. Cells are instructed to elongate and align mediolaterally (Jessen et al., 2002; Kilian et al., 2003; Topczewski et al., 2001; Wallingford et al., 2000). Protrusive activity is oriented mediolaterally, and protrusions in favored directions are selectively stabilized (Wallingford et al., 2000). Together, these and other modifications contribute to persistent directed movement. Cell divisions are aligned in the dorsal half of the embryo so that daughter cells contribute to extension along the anterior–posterior axis (Gong et al., 2004). The range of cellular effects suggests that many downstream pathways may be regulated.

PCP/Wnt signaling is believed to involve a "core cassette" of genes that is composed of Frizzled, Dishevelled, Prickle, and Strabismus/Vangogh-like 2. In vertebrates (and in contrast with *D. melanogaster*), PCP/Wnt pathway also employs the ligands Wnts 4, 5, and 11 (Heisenberg et al., 2000; Ungar et al., 1995; Westfall et al., 2003), and it is thought to function primarily without transcriptional output (Figure 17.7). Wnt ligands signal from the receptor Frizzled to Dishevelled, a multidomain protein that appears to integrate signals and modify cell functions through a large number of binding partners (see Wallingford and Habas, 2005). Dishevelled must move from the cytoplasm to the cell membrane to participate in PCP/Wnt signaling (Park et al., 2005). Strabismus/Vangogh, a multipass membrane protein, and Prickle, an intracellular protein, appear to act antagonistically to PCP/Wnt signaling (Klein and Mlodzik, 2005).

Effects on cell elongation and migration suggest that PCP/Wnt signaling controls the actin cytoskeleton. One downstream effector of Dishevelled is Dishevelled associated activator of morphogenesis 1 (DAAM1), which has been proposed to directly link Dishevelled with the activation of the small GTPase Rho (Habas et al., 2001). Rho, in turn, activates its effectors, Rho kinase (Rok; Marlow et al., 2002) and Diaphanous, to regulate the actin cytoskeleton (Habas et al., 2003; Zhu et al., 2006). As formin homology proteins, DAAM1 and Diaphanous may directly nucleate the formation of linear actin (Zigmond, 2004). Dishevelled stimulates a second pathway through the small GTPase Rac, which can regulate the formation of lamellipodia (Zigmond, 2004). Rac stimulates the Jun N-terminal kinase, which can phosphorylate proteins and turn on transcription from a reporter c-Jun promoter.

An additional noncanonical Wnt pathway known as the Wnt/calcium (Wnt/Ca) pathway has been implicated in C&E movements (Kohn and Moon, 2005; Kuhl, 2004). There is considerable overlap between Wnt/Ca and Wnt/PCP in terms of molecular components and readouts. Both involve Wnts 5 and 11, and both may involve Dishevelled. Both stimulate the release of

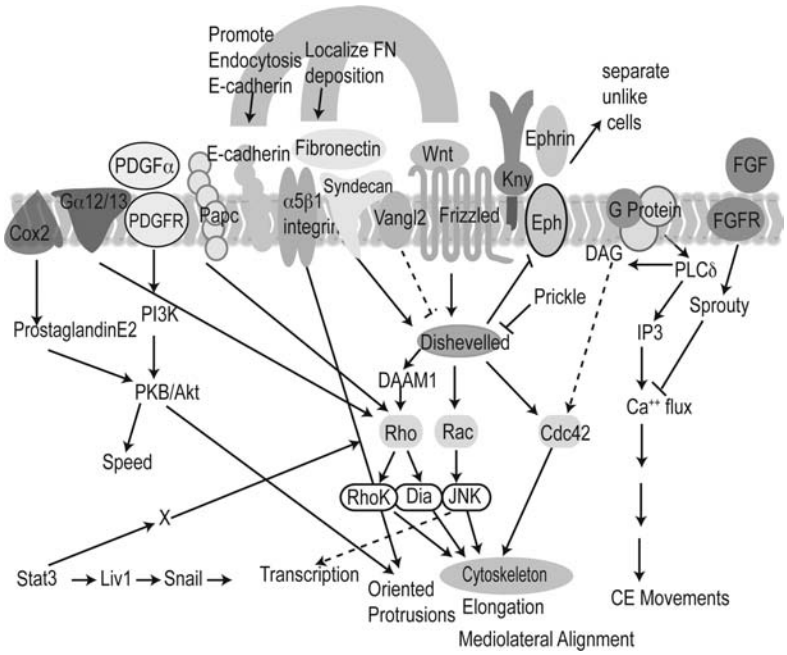

FIGURE 17.7 **Molecules that affect convergence and extension.** Noncanonical Wnt signaling through planar cell polarity/Wnt and Wnt/calcium is central to our understanding of convergence and extension. Wnt signaling interacts with fibronectin/integrin signaling and the adhesion systems that involve cadherin, protocadherin, and ephrins. Stat3, platelet-derived growth factor, Gα12/13, and some fibroblast growth factor signaling are (so far) largely independent of Wnt signaling. (See color insert.)

intracellular calcium, and both have effects on cell movement. Frizzled receptors have a seven transmembrane domain structure similar to G-protein–coupled receptors (Malbon, 2005). In the Wnt/Ca pathway, Frizzled interacts with heterotrimeric G proteins to turn on phospholipase C and its downstream effectors IP_3 and DAG. IP_3 stimulates calcium release from intracellular stores, thereby activating calcium-sensitive enzymes (Sheldahl et al., 1999; Slusarski et al., 1997a; Slusarski et al., 1997b). Wnt11 signaling activates the small GTPase Cdc42. This is blocked by pertussis toxin, which suggests that Cdc42 is downstream of G proteins and a target of Wnt/Ca signaling (Choi and Han, 2002; Kim and Han, 2005; Penzo-Mendez et al., 2003). Wnt signaling regulates several signaling pathways to control the actin and microtubule cytoskeletons and in turn to drive cell movements and to shape change.

5. Cell-Adhesion Molecules

Essential to motility is the fact that cells must dynamically control both cell–cell and cell–matrix adhesion. Apart from its role in epiboly (described previously), several studies using zebrafish indicate that E-cadherin plays a role in the C&E of both the ectoderm and mesoderm. The loss of E-cadherin impairs C&E, causing a shorter, wedge-shaped, body axis; a fragmented prechordal mesoderm; and broadened ectodermal structures, which can be seen by mid gastrulation (Babb and Marrs, 2004; Kane et al., 2005; Shimizu et al., 2005). Later, as cells accumulate at the dorsal midline, their cohesiveness increases, and they form an epithelium-like tissue. When E-cadherin

function is reduced, somites and the neural tube are expanded mediolaterally, and somite borders form irregularly; this is associated with defective convergence movements (McFarland et al., 2005).

Adhesion over the surface of a motile cell must change with cycles of attachment to, movement over, and release from the substratum. Consistent with the notion of dynamic regulation of adhesion is that the removal of E-cadherin from the cell membrane by endocytosis is required for C&E, and it is stimulated by PCP/Wnt signaling (Ulrich et al., 2005). Presumably, the localized addition of E-cadherin molecules is also regulated.

In addition, mesodermal cells must maintain a level of "mesoderm" adhesion to prevent them from merging back into the ectoderm or into other tissues that are separating away. In embryos with reduced E-cadherin expression, mesoderm fails to separate well from the overlying ectoderm (Montero et al., 2005). Adhesion molecules with wide expression profiles like E-cadherin may be responsive to many signaling pathways. What these pathways are and how signals are integrated require further study.

6. Protocadherin 1, Paraxial Protocadherin, Papc

Protocadherin 1, paraxial protocadherin, Papc (Pcdh1) is a member of a family of adhesion molecules related to the cadherins (see Chapter 18). Pcdh1 is expressed in a more limited range of cells, primarily the presomitic mesoderm (Kim et al., 1998; Yamamoto et al., 1998). The knockdown of Pcdh1 impairs the mediolateral alignment of mesodermal cells and reduces their ability to intercalate and converge. Pcdh1 acts by activating Rho and Jun N-terminal kinase to alter the actin cytoskeleton in parallel to or downstream of Wnt signaling (Unterseher et al., 2004). In addition, Pcdh1 regulates the activity of C-cadherin (Chen and Gumbiner, 2006). Adhesion molecules with more restricted expression patterns may offer a way to regulate movements in specific tissues.

7. Ephrin/Eph

The Ephrin and Eph signaling pathway is involved in the sorting and separation of adjoining cell populations and tissues, such as somites and brain rhombomeres, by regulating cell–cell adhesion (see Pasquale, 2005, and chapter 22 by Daar in this book). Ephrin/Eph assists the migration of cells during gastrulation. The overexpression of soluble EphrinB2 disturbs signaling and inhibits convergence movements (Chan et al., 2001). Wnt signaling may modulate Ephrin-mediated cell sorting. Dishevelled can bind EphrinB and EphB receptors through linker molecules, and it can block cell sorting mediated by EphB (Lee et al., 2006; Tanaka et al., 2003). How Ephrins support orderly cell movements, particularly the cell behaviors regulated during C&E, remain open questions.

8. Fibronectin/Integrin/Syndecan-4

During gastrulation, glycoproteins form an extracellular matrix that surrounds cells and that is deposited between the germ layers, effectively separating the mesoderm from the ectoderm and the endoderm. Fibronectin and its receptors, integrin and syndecan, are prominent components of this matrix, and they mediate both adhesion and signaling. Studies using amphibians showed that fibronectin is essential for several gastrulation movements

(Boucaut et al., 1990; Winklbauer et al., 1996), including epiboly, which was discussed previously (Longo et al., 2004). In *X. laevis,* the migration of the head mesoderm and the extension of the lateral plate mesoderm require contact between mesoderm and fibronectin on their movement substratum, the blastocele roof. Convergent extension of the axial mesoderm requires that fibronectin be deposited on the outside of the mass of the axial mesoderm.

Several studies in *X. laevis* show that fibronectin/integrin signaling modulates adhesion and protrusive activity during convergent extension. Fibronectin/integrin signaling positively regulates cadherin binding between mesodermal cells (Marsden and DeSimone, 2003). Fibronectin limits and orients the formation of lamellipodia. Cells lacking fibronectin are untidy; they form an increased number of protrusions, and they fail to elongate and mediolaterally align. Interestingly, their elongation can be rescued by contact with a fibronectin-coated substratum. Fibronectin may act to repress protrusions that are directed out of the tissue or that are oriented away from the midline. Thus, fibronectin may channel cell movement in a direction that supports convergent extension (Davidson et al., 2006).

Fibronectin and PCP/Wnt signaling influence each other. PCP/Wnt signaling promotes the deposition of fibronectin at tissue boundaries. In the absence of PCP/Wnt signaling, fibronectin is found within the mesodermal tissue. However, Wnt11 function is also thought to be needed for normal adhesion to fibronectin (Puech et al., 2005). Signaling can proceed in the opposite direction as well. Contact with fibronectin can rescue cell elongation when some PCP/Wnt components (Frizzled and Strabismus/Vangoghlike2) but not others (Prickle) are inactive; this suggests that some steps (Frizzled and Strabismus/Vangoghlike2) are upstream of fibronectin signaling (Goto et al., 2005).

The heparan sulphate proteoglycan syndecan-4 may link fibronectin to Wnt signaling and Dishevelled activation. Normal levels of syndecan-4, which is a fibronectin receptor, are required for C&E in *X. laevis*. Fibronectin binding is needed for Dishevelled to become active (Marsden and DeSimone, 2001), but fibronectin cannot bind Dishevelled directly (Munoz et al., 2006). Interestingly, syndecan-4 brings together a complex of fibronectin, Wnt, Frizzled, and Dishevelled at the cell membrane to activate the PCP/Wnt pathway (Munoz et al., 2006). The interactions between PCP/Wnt signaling and cell–cell and cell–matrix adhesion systems are likely to be the targets of many other molecules and a useful framework for organizing the molecular regulation of C&E.

9. Heterotrimeric G Proteins: α12/13

G proteins of the α12/13 subclass are known for their role in the chemotaxis of mammalian neutrophils and epithelial folding during *D. melanogaster* gastrulation (Leptin, 2005; Van Haastert and Devreotes, 2004). Recent studies implicated them in several aspects of cell motility during gastrulation and showed that they likely act in parallel with noncanonical Wnt signaling (Lin et al., 2005). G proteins transduce signals from G-protein–coupled receptors. The activation of the G protein stimulates it to separate into α and $\beta\gamma$ subunits, each of which turns on downstream effectors. The Gα12/13 subclass regulates the activity of the small GTPase Rho (Goulimari et al., 2005).

Zebrafish embryos depleted of Gα12 and Gα13 proteins exhibit C&E defects that become apparent at mid gastrulation. Dorsally migrating

mesodermal cells proceed with reduced speed and rounder cell morphology. The ability to migrate directionally is further reduced by an increased rate of turning and a reduced ability to correct an errant course. In addition, Gα12/13 function is needed cell-autonomously for notochord cells to elongate and perform efficient mediolateral intercalation. Gα12/13 is likely to act in parallel with noncanonical Wnt signaling, because cell elongation and C&E defects are exacerbated when both Gα12/13 and Wnt signaling are inhibited (Lin et al., 2005). How Gα12/13 proteins are activated during gastrulation and in their downstream signaling pathways is not known.

10. Effectors of FGF

As discussed previously, FGFs regulate cell fate and motility during gastrulation. The motility functions of FGF could be distinguished from patterning functions after distinct downstream effectors of motility were found. Among these are Sproutys, neurotrophin-receptor–related homolog, G-protein–coupled receptor 4, *X. laevis* marginal coiled protein, and Polycomb group gene ph2α (Chung et al., 2004; Chung et al., 2005; Frazzetto et al., 2002; Komoike et al., 2005; Nutt et al., 2001; Sasai et al., 2004; Sivak et al., 2005).

Sproutys and Spreds are structurally related molecules that regulate the inductive and morphogenetic functions of FGF signaling. Sproutys are expressed early and block morphogenesis by inhibiting the Wnt/Ca pathway while sparing the inductive functions. Spreds, on the other hand, are expressed later and block induction without effecting movements (Nutt et al., 2001; Sivak et al., 2005). In this way, FGF signaling is first channeled toward induction and later channeled toward the modulation of movement.

D. The Coordination of Anterior–Posterior and Dorsal–Ventral Axes and Convergence and Extension

How are the embryonic axes coordinated so that the body axis is aligned with the direction of extension? We have only a limited understanding of this process, and, as one might expect, it appears to involve several layers of regulation. Anterior–posterior polarity controls the direction of C&E so that cells elongate and mediolaterally intercalate perpendicular to the direction of embryo elongation. Anterior–posterior pattern in mesodermal tissue can be revealed by the genes *chordin* and *Xbra,* which are expressed in opposing anterior–posterior gradients. Explants composed of just anterior or just posterior mesoderm fail to elongate. However, explants of anterior mesoderm placed against posterior mesoderm elongate (Ninomiya et al., 2004). The juxtaposition of strongly different anterior–posterior levels is not necessary, because explants with smoothly graded anterior–posterior levels also elongate, and this is the more natural situation (Ninomiya et al., 2004). The molecular mechanisms that translate the anterior–posterior positional information into C&E cell behaviors remain to be determined.

Moreover, BMPs are thought to coordinate the patterning of cell fates and C&E movements along the dorsal–ventral gastrula axis (Myers et al., 2002a). As discussed previously and in detail in other chapters of this book, the ventral-to-dorsal gradient of BMP activity forms with a low point near the SMO. BMPs work as morphogens to specify ventral and posterior cell fates and to inhibit dorsal–anterior fates in all germ layers. Genetic studies in zebrafish

demonstrated that thresholds of BMP activity specify distinct domains of C&E. At high BMP activity levels in the ventral gastrula region, C&E movements do not occur; at moderate BMP activity levels, strong C&E driven by directed migration take place; at low BMP activity levels near the SMO, strong extension and moderate convergence are observed, and these are driven by mediolateral intercalation.

Do these anterior–posterior and dorsal–ventral patterning systems regulate gastrulation cell movement behaviors downstream of or in parallel with their effects on cell fates? An intriguing possibility is that parallel mechanisms downstream of BMP signaling exist to regulate cell fates and cell movements (Myers et al., 2002b). In zebrafish paraxial mesoderm, a different threshold of BMP regulates the expression of the *wnt5* gene, which is necessary for C&E movements, than the threshold that regulates the expression of the *myoD* gene, which encodes a cell-fate regulator (Myers et al., 2002a).

VII. CONCLUSION

The complexity and dynamic nature of vertebrate gastrulation have both fascinated scientists and proved challenging experimentally. The cellular and molecular mechanisms of gastrulation are now being revealed with new methodologies. Imaging techniques link tissue morphogenesis to specific gastrulation cell behaviors *in vivo,* whereas developmental genetics uncovers the molecules that regulate individual gastrulation cell behaviors and underlying cellular properties. Forward genetic screens in fish and mouse will continue to identify molecules with essential roles during gastrulation. The growing complement of reverse genetic tools provides the means of testing the roles of additional molecules during gastrulation.

SUMMARY

- Gastrulation is a fundamental developmental process that establishes the triploblastic body plan with three germ layers (mesoderm, endoderm and ectoderm) that are subsequently shaped into the head, trunk, and tail.
- Vertebrate gastrulation is accomplished by four evolutionarily conserved gastrulation movements: internalization, epiboly, convergence, and extension.
- During internalization, the germ layers form as endodermal cells move via the blastopore to take the deepest position, and mesodermal cells move between the endodermal and the ectodermal layers.
- Gastrulation movements are achieved by a combination of cell behaviors, including directed cell migration, cell intercalations, cell shape changes, and proliferation.
- Despite morphologic differences, similarities emerge with respect to the cellular and molecular genetic mechanisms underlying gastrulation in different vertebrate groups.
- The internalization of mesoderm entails some aspects of EMT in different vertebrates. This process requires the downregulation of the cell-adhesion molecule E-cadherin via the Snail transcriptional repressor. The function

of Snail is tightly regulated by many pathways, including FGF, p38 NIK, and Stat3.

- The patterns of cell movements with respect to the blastopore and the SMO are remarkably similar among vertebrates. At the onset of gastrulation, the internalized mesodermal cells move away from the blastopore. Later during gastrulation, trajectories of the mesodermal cells turn toward the nascent axial mesoderm in the embryo midline to initiate convergence movements.
- During chick gastrulation, FGF signaling is thought to regulate the movement of mesodermal cells away from the blastopore and later to attract them toward the embryonic midline.
- In zebrafish gastrulae, the Stat3 transcription factor is activated in the nascent embryonic midline, where it regulates the C&E of the midline tissues and promotes the production of secreted factor(s) that promote the convergence of lateral cells toward the midline.
- The noncanonical Wnt signaling pathway is required for a number of cell behaviors that drive C&E movements, including directed cell migration, mediolateral intercalation, and polarized radial intercalation.

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RECOMMENDED RESOURCES

The Virtual Embryo:
http://www.ucalgary.ca/UofC/eduweb/virtualembryo/db_tutorial.html

The Virtual Library of the Society for Developmental Biology:
http://www.sdbonline.org/archive/Other/VL_DB.html

18

REGULATION OF TISSUE SEPARATION IN THE AMPHIBIAN EMBRYO

HERBERT STEINBEISSER

Institute of Human Genetics, University Heidelberg, Heidelberg, Germany

INTRODUCTION

During embryogenesis, tissues are specified, and they become differentiated from their neighbors (Heasman, 2006). To establish and maintain the embryonic body plan, separated tissues may not mix. Therefore, groups of cells or tissues must develop separation behaviors that allow for the formation of defined boundaries. Cell sorting and tissue separation behavior in the early embryo is complicated by the extensive cell movements that constantly confront the cells with new microenvironments. The earliest separation behavior in the Xenopus embryo is observed when the mesendoderm involutes and remains separated from the neuroectoderm. As a result, a border called *Brachet's cleft,* which shows fibronectin deposition, is formed between the mesendoderm and the ectoderm (Figure 18.1). The anterior domain of Brachet's cleft is generated by vegetal rotation whereby the anterior endoderm actively moves toward the blastocele roof (BCR; Winklbauer and Schurfeld, 1999; Wacker et al., 2000). The posterior cleft develops when the mesoderm invaginates through the blastopore lip and the anterior–posterior axis of the embryo forms. To achieve tissue separation, the ectoderm must develop a repulsive behavior. The formation of an interface between the mesendoderm and the ectoderm is essential for the ability of these tissues to pass each other during gastrulation. The molecular mechanisms that control the different aspects of tissue separation are not fully understood. The Xenopus embryo is particularly suited to address the phenomenon of tissue separation experimentally. Ectoderm and mesendoderm are not separated by a basal lamina, which could act as an insulator between these tissues. The fibronectin fibrils on the BCR form only a loose network, which is not able to physically separate the ectoderm and the mesendoderm. These cell layers are in close contact, which

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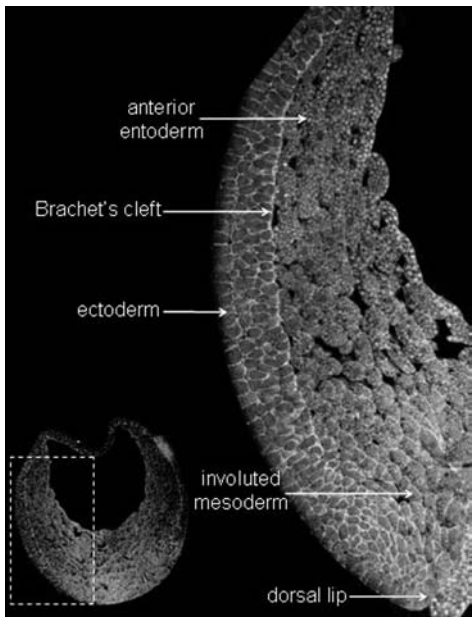

FIGURE 18.1 Brachet's cleft marks the border between the ectoderm and the mesendoderm. A histologic section of a Xenopus embryo at the gastrula stage stained for X/BU cadherin (*green*), Fibronectin (*red*), and DAPI (*blue*). Fibronectin deposition marks the border between the ectoderm and the mesendoderm. Cadherin is localized to the cell membranes, and DAPI stains the nuclei. (Photograph courtesy of Dr. T. Kurth. See color insert.)

indicates that separation and repulsion are intrinsic properties of the cells. Another important advantage of the Xenopus system is the availability of *in vitro* assays that allow for the experimental analysis of separation and repulsion in tissues in a defined time frame. This review will summarize the experimental work on tissue separation that has been published in the Xenopus system and discuss the general relevance of the proposed cellular mechanisms involved in this morphogenetic process.

On the basis of the observation that cells in the involuting mesoderm do not mix with BCR tissue, Winklbauer and Schurfeld (1999) developed an assay system that allowed for the systematic experimental analysis of tissue separation (Wacker et al., 2000). The BCR assay consists of positioning cell aggregates from different regions and different developmental stages of early Xenopus embryos on BRC explants and scoring their ability to separate (Figure 18.2). Wacker et al. (2000) were able to show that the endoderm already displays separation behavior during the blastula stages (stage 8.5). However, the dorsal mesoderm first develops separation behavior when the cells invaginate through the blastopore during gastrulation (stages 10 and 11). At the same time, repulsive behavior by ectodermal tissue against mesendoderm peaks (stages 10–10.5; Wacker et al., 2000). These experiments demonstrate that the development of tissue separation in the early Xenopus embryo is temporally controlled and that all three germ layers contribute to this morphogenetic cell behavior.

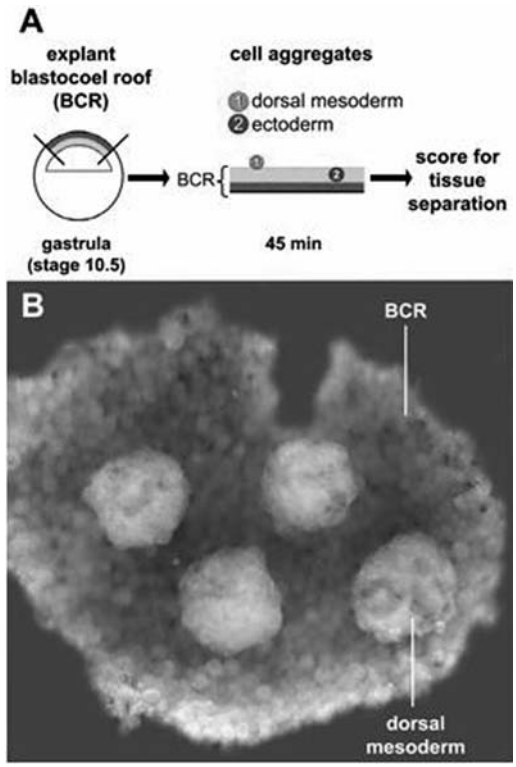

FIGURE 18.2 **The blastocele roof (BCR) assay allows one to test separation behavior in embryonic tissues. A,** Schematic summary of the BCR assay. BCR tissue is explanted during the gastrula stage (stage10.5), and cell aggregates to be tested for separation behavior are placed on the BCR tissue. Separation behavior is scored after 45 minutes. Dorsal mesoderm (*red*) separates from the BCR, but ectoderm (*blue*) integrates. **B,** Cell aggregates derived from the dorsal marginal zone do not integrate into the BCR tissue as a result of the repulsion of the ectoderm and the separation behavior of the dorsal mesoderm. (See color insert.)

I. THE ROLE OF MESODERM-INDUCING GROWTH FACTORS AND CADHERIN-MEDIATED TISSUE AFFINITIES

Cells of the involuting dorsal mesoderm do not mix with the ectoderm; therefore, one could argue that separation behavior is a component of "mesoderm identity." This view is supported by tissue separation experiments in which mesoderm formation was induced or inhibited. Mesoderm can be induced in blastula animal cap cells by activin-like transforming growth factor beta (TGF-β) proteins or fibroblast growth factors (FGFs; Green, 2002). Mesodermal tissue with dorsal character induced by activin displays tissue separation, but FGF-induced ventral mesoderm fails to separate from ectoderm (Wacker et al., 2000). When the FGF pathway is blocked in activin-treated animal caps by a dominant negative FGF receptor, separation behavior is lost. These data indicate that that FGF signaling is required but not sufficient for the tissue separation of ectoderm and mesoderm. TGF-β not only induces cell fate changes in animal cap tissue, but it also influences the adhesive properties of the cells.

It is tempting to speculate that differences in cell adhesion in the mesendoderm and the ectoderm are the driving forces behind the phenomenon of

tissue separation in the Xenopus gastrula. Well-documented examples exist to demonstrate that, indeed, cell sorting and the formation of tissue borders can be attributed to differences in adhesive properties.

In 1907, Wilson reported that dissociated cells of marine sponges were able to reconstitute functional organisms. The phenomenon of cell sorting and tissue reconstitution in amphibian embryos was intensively studied and described by Holtfreter in publications that appeared between 1939 and 1955. The central observation was that dissociated and mixed embryonic tissues reaggregate randomly but then self-sort and often assume positions that reflect the correct anatomic tissue arrangement in the embryo. Holtfreter explained this effect by postulating specific "tissue affinities" (Gewebsaffinitäten) that mediate attraction and repulsion (Holtfreter, 1939). The reconstitution of tissues from mixed aggregates thus requires specific tissue affinities as well as sorting, both of which are dependent on cell motility. All of these components were incorporated into the differential adhesion hypothesis formulated by Steinberg (1996), which served as a conceptional basis for the explanation of morphogenetic events during embryogenesis. During the last 20 years, the cellular adhesion systems have been identified and characterized. This enables us to now analyze the role of specific cell adhesion molecules in the regulation of morphogenetic cell behaviors.

Transmembrane proteins of the cadherin family are essential components of the cell adhesion machinery that mediate homophilic cell–cell adhesion and that contribute to cell sorting (Figure 18.3; Schambony et al., 2004; Steinberg, 1996; Tepass et al., 2002). It has been demonstrated in cell culture systems that quantitative differences in cadherin protein levels are sufficient to separate cell populations (Steinberg and Takeichi, 1994). This mechanism was found to be important *in vivo* as well. For example, in the Drosophila system, the level of E-cadherin regulates cell sorting and determines the position of the oocytes in the ovary (Godt and Tepass, 1998).

Cadherin-mediated cell adhesion also plays a role in tissue separation in Xenopus. The overexpression of XB/U or EP/C-cadherins in involuted mesoderm and in TGF-β–induced animal cap tissue abolishes separation from the BCR. By contrast, the inhibition of cadherin function by the expression of dominant negative cadherin proteins in the BCR does not affect the separation behavior of mesoderm cells, which suggests that this morphogenetic behavior cannot be exclusively explained by differential cell adhesion. Interestingly, blocked cadherin function in uninduced animal cap (AC) cells inhibits their integration into untreated BCR tissue, which indicates that tissue separation is not directly linked to mesoderm and endoderm differentiation (Wacker et al., 2000). This implies that morphogenetic cell behaviors and patterning mechanisms can be experimentally separated.

II. THE ROLE OF TRANSCRIPTION FACTORS IN TISSUE SEPARATION

As detailed above, the stimulation of AC cells with FGF is not sufficient to induce tissue separation. When the transcription factors Mix1 and Goosecoid (gsc) are expressed in addition to FGF treatment, however, separation behavior is induced. In the embryo, Mix1 and gsc expression are induced by TGF-β signaling, and both proteins regulate the transcription of genes in the mesoderm and the endoderm (Cho et al., 1991; Latinkic and Smith, 1999;

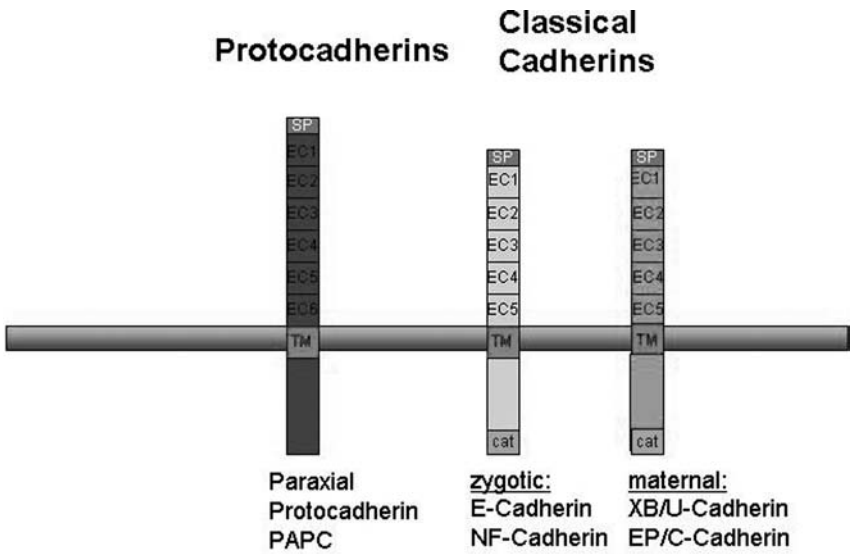

FIGURE 18.3 **Structures of classical cadherins and of paraxial protocadherin.** The proteins contain signal peptides (sp), extracellular cadherin domains (EC), single transmembrane domains (TM), and cytoplasmic domains. Classical cadherins but not paraxial protocadherins possess a catenin binding site (cat) at the C-terminus. XB/U and EP/C cadherins are provided maternally; they regulate adhesion in the early embryo.

Niehrs et al., 1993; Wacker et al., 2000). In addition, Mix1 and gsc are involved in the regulation of morphogenesis. Mix1 modulates cell adhesion and the ability of cells to form protrusions, thereby allowing for the polarization of cells, and it is crucial for cell migration. Alternatively, gsc induces migratory behavior and regulates the movement of prechordal plate tissue during gastrulation (Niehrs et al., 1993). The activity of both factors is required in the mesoderm for tissue separation, and antimorphic gsc and Mix1 proteins compromise the separation of dorsal mesoderm from ectoderm. Neither the gsc and Mix1 target genes that are relevant in this context nor the responsible molecular mechanisms are known.

A third transcription factor, Lim1, was also shown to play a role in tissue separation. When this component of the Spemann organizer is inhibited in Xenopus embryos, involution of the mesoderm and the separation of mesendoderm and ectoderm is also inhibited (Hukriede et al., 2003). One target of Lim1 is the paraxial protocadherin (PAPC) gene (Kim et al., 1998). Protocadherins are transmembrane proteins that mediate homophilic cell–cell interactions via their extracellular domains, and they promote cell sorting (Redies et al., 2005). This finding provides a link between the regulation of transcription by Lim1 and a component of the cellular adhesion machinery. In Xenopus embryos, PAPC is expressed in the organizer and later in the paraxial mesoderm, where it contributes to the formation of the somites (Kim et al., 1998; Yamamoto et al., 1998). PAPC expression is reduced in Xenopus embryos with knocked-down Lim1 function, in $Lim1^{-/-}$ mice, and in *spade tail* zebrafish mutants (Hukriede et al., 2003; Warga and Nusslein-Volhard, 1998; Yamamoto et al., 1998). Taken together, these results show that tissue separation requires the activities of the transcription factors gsc, Mix1, and Lim1, which are all expressed during gastrulation in the dorsal mesendoderm. The means by which

these proteins regulate the components of cell adhesion systems and influence the signaling pathways that modulate morphogenesis are not yet clear.

III. THE ROLE OF SIGNALING MOLECULES IN TISSUE SEPARATION

A. Frizzled Mediated Signaling

Morphogenetic cell behaviors and movements in the embryo are regulated by defined signaling pathways. Experiments in invertebrate and vertebrate embryos have identified Wnt signaling as a major player in the regulation of morphogenesis. Wnt/β-catenin signaling is essential in the Xenopus embryo to establish the border between notochord and somites. Interestingly, cadherin-mediated cell adhesion does not play a role the formation of this tissue boundary (Reintsch et al., 2005).

Planar cell polarity (PCP) in Drosophila is regulated by the so-called noncanonical Wnt signaling, which does not require β-catenin function (Bejsovec, 2005; Logan and Nusse, 2004; Schambony et al., 2004). The PCP pathway does not promote cell fate changes; rather, it regulates cell shape and morphogenic behaviors. This requires the connection to and the modulation of the cytoskeleton. Rho and c-Jun terminal kinase (JNK) are such modulators of the cytoskeleton, and they are components of the PCP signaling pathway that regulates cell polarization and convergent extension movements during gastrulation in vertebrates (Wallingford et al., 2000; Wallingford and Harland, 2001; Fanto and McNeill, 2004).

The Frizzled (Fz) family of receptors is a key component of the PCP pathway in Drosophila as well as in vertebrates. These seven transmembrane domain receptors interact with secreted glycoproteins of the Wnt family, and they can activate the Wnt signaling cascade(s) (Logan and Nusse, 2004). The Xenopus Fz7 receptor can interact with ligands of the Wnt-1 type, such as Wnt-8b, and with those of the Wnt-5a type, such as Wnt-5a and Wnt-11 (Medina et al., 2000; Djiane et al., 2000; Sumanas et al., 2000). Fz7 is also able to act in different branches of the Wnt signaling cascade. This receptor can activate the Wnt/β-catenin, the PCP, and the Ca^{++}/protein kinase C (PKC) branches of the Wnt pathway (Medina et al., 2000). In the early Xenopus embryo, Fz7 is expressed preferentially on the dorsal side, and the mRNA is found in the ectoderm, the mesoderm, and the endoderm. The knockdown of Xfz7 function using antisense Morpholino (Mo) oligonucleotides causes a loss of the posterior part of Brachet's cleft, which indicates that tissue separation of the invaginating mesoderm and the ectoderm is disturbed. This finding is supported by results using the *in vitro* BCR assay (Winklbauer et al., 2001). When cells excised from the dorsal mesoderm are placed on BCR, no mixing of these cell populations occurs. However, the mesoderm cells integrate into the ectoderm when Xfz7 function is knocked down in the dorsal mesoderm. Further experiments have demonstrated that Xfz7 activates PKC in a G-protein–dependent manner and that PKC can rescue separation behavior in Mo Xfz7-injected dorsal mesoderm. Activation of the Wnt/β-catenin or the PCP pathways is not sufficient to rescue the MoXfz7-induced defects (Winklbauer et al., 2001).

These experiments identified the Xenopus Fz7 receptor as a crucial component of the tissue separation machinery. In this context, Xfz7 does not

activate the PCP pathway; rather, it triggers the Ca^{++}/PKC branch of the Wnt signaling cascade.

At this point, no Wnt ligand involved in tissue separation has been identified, but recently it was shown that the extracellular domains of Xfz7 and PAPC can interact. Combined gain-of-PAPC and -Xfz7 function in AC tissue induces separation behavior, despite the lack of mesoderm induction. The coexpression of a secreted extracellular domain of Xfz7, which interferes with the XFz/PAPC interaction, results in a loss of separation behavior, which indicates that this interaction is biologically relevant (Medina et al., 2004).

B. PAPC Signaling Function in Tissue Separation

The importance of PAPC in the regulation of tissue separation and convergent extension movements has been demonstrated in Xenopus embryos. Gain-of-PAPC function rescued the gastrulation defects in Lim1-depleted embryos (Hukriede et al., 2003). Antisense Mo-induced knockdown of PAPC function impairs the formation of the posterior part of Brachet's cleft, and it inhibits both convergent extension movements and separation behavior in dorsal marginal zone explants and in TGF-β–induced ACs (Medina et al., 2004; Unterseher et al., 2004). The PAPC knockdown phenotype is very similar to that seen in Mo Xfz7-injected embryos, but the Mo PAPC effect could not be rescued by Xfz7 and vice versa. This clearly indicates that these two molecules have nonredundant functions in tissue separation.

Another interesting finding was that the adhesive properties of PAPC, which are required for cell sorting, are not needed for tissue separation. When Xfz7 and M-PAPC (a membrane-tethered, truncated protein with strong adhesive properties) are expressed in ACs, no separation behavior is observed (Medina et al., 2004).

Protocadherins are not only modulators of cell adhesion, but they are also mediators of intracellular signaling (Redies et al., 2005) One could therefore hypothesize that protocadherins, in addition to their adhesive properties, exert signaling functions that could contribute to PCP signaling. Such a connection between protocadherins and the PCP pathway has recently been described in Drosophila, whereby the protocadherins FAT and Dachsous are involved in the regulation of cell polarity (Cho and Irvine, 2004).

In addition to its adhesive function, Xenopus PAPC is able to activate Rho and JNK, which are mediators of PCP signaling (Medina et al., 2004; Unterseher et al., 2004). A gain of PAPC resulted in the activation of Rho and JNK in ventral marginal zone and AC explants. The expression of a dominant negative PAPC protein or Mo PAPC reduces the level of guanosine 5'-triphosphate-bound Rho in dorsal marginal zone explants (Hukriede et al., 2003; Medina et al., 2004; Unterseher et al., 2004). In the context of tissue separation, PAPC exerts a mechanistically as yet uncharacterized signaling function, whereas its adhesive properties do not seem to play a role in this morphogenetic behavior. Because Xfz7 and PAPC have nonredundant functions in the development of tissue separation, one could speculate that this process requires balanced levels of both PKC and Rho/JNK activity (Figure 18.4). Further experiments will need to focus on the mechanism(s) of PAPC-mediated signaling and on the role of the interaction between Xfz7 and PAPC.

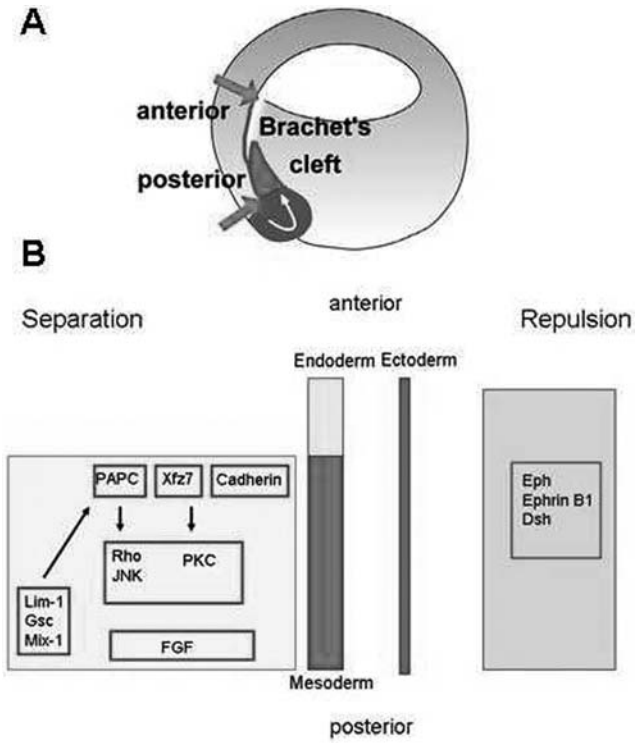

FIGURE 18.4 **Molecules and pathways involved in the formation of Brachet's cleft. A,** The anterior part of the cleft is formed between the endoderm (*light gray*) and the ectoderm (*dark gray*). The posterior cleft arises when the dorsal mesoderm (*medium gray*) involutes through the blastopore lip. **B**, A summary of the molecules involved in the repulsion behavior of the ectoderm and the separation behavior of the dorsal mesoderm. Eph/EphrinB1 (Tanaka et al., 2004; Lee et al., 2005); PAPC (paraxial protocadherin; Medina et al., 2004); Xfz7 (*Xenopus* frizzled 7; Winklbauer et al., 2001; Medina et al., 2004); cadherins (Wacker et al., 2000); goosecoid (gsc), mix-1 (Wacker et al. 2000); lim-1 (Hukriede et al 2003); Rho, JNK (c-Jun terminal kinase; Tanaka et al., 2004; Medina et al. 2004); PKC (protein kinase C; Winklbauer et al., 2001); FGF (fibroblast growth factor; Wacker et al., 2000); Dsh (disheveled; Tanaka et al., 2003; Lee et al., 2005). The mechanism that regulates the separation of the endoderm and the ectoderm is not known.

C. Ephrin/Eph-Mediated Separation Behavior

Signaling events mediated by Ephrin/Eph regulate cell adhesion and repulsion (see Chapter 21). There is experimental evidence that this signaling module also plays a role in the development of repulsion behavior in the Xenopus ectoderm.

Eph receptors are tyrosine kinases that interact with their membrane-bound Ephrin ligands. The cytoplasmic domain of the Eph receptors can bind src-homology 2 (SH2) domain proteins, which regulate the cytoskeleton (Kullander and Klein, 2002). The interaction of Rho guanidine exchange factors and Eph A proteins connects Eph signaling to the Rho-mediated modulation of cell motility (Mellitzer et al., 2000; Shamah et al., 2001). Upon ligand binding, the receptor-bearing cells develop repulsive behavior, which can result in the formation of tissue boundaries. In cells that express the ephrin ligands, so-called "reverse signaling" can be observed. The C-terminus of

the ephrins is phosphorylated by src kinase, and regulators of the cytoskeleton such as p21 activated kinase PAK1 are recruited (Cowan and Henkemeyer, 2001; Palmer et al., 2002). The role of Ephrin/Eph signaling in tissue separation was clearly shown in the zebrafish somite and hindbrain (Cooke et al., 2001; Durbin et al., 1998). In these structures, actual clefts form as a result of EprinB2/EphA4 signaling, and no cell mixing can occur across these boundaries. The sorting of dissociated and mixed cells from Xenopus and zebrafish blastula embryos is induced by Ephrin/Eph signaling (Mellitzer et al., 1999; Tanaka et al., 2003). When tissue separation of EphrinB1 and Eph B2-expressing Xenopus ectoderm and BCR tissue was tested, at least one aspect of separation behavior was observed. Inner cells of the AC expressing EphrinB1 or Eph B2 sink into BCR tissue that is expressing the corresponding receptor/ligand, but the cell populations do not intermingle (Tanaka et al., 2003). This indicates that the Ephrin/Eph cassette is not sufficient to elicit complete separation behavior, but it prevents the mixing of cells with the surrounding BCR tissue. Recent publications also provide evidence that there is crosstalk between Ephrin/Eph signaling and the PCP pathway. Ephrin/Eph-induced cell sorting and the prevention of cell mixing is dependent on disheveled (Dsh) function. The cytoplasmic protein Dsh is an effector of Wnt/β-catenin signaling and of the PCP pathway. Both Eph receptors and Ephrins physically interact with the DEP domain of the Dsh protein, which mediates noncanonical (PCP) signaling. Forward and reverse Ephrin/Eph signaling therefore involves Dsh function. The Wnt/β-catenin pathway, however, does not seem to play a role in Ephrin/Eph-mediated cell sorting. The overexpression of a Dsh mutant, which lacks the DIX domain and is incapable of mediating Wnt/β-catenin signaling, does not affect cell sorting. In agreement with these results is the finding that Eph/Ephrin induces the activation of PKCδ, the small GTPase Rho, and Rho kinase, is dependent on Dsh, and requires the DEP domain. Accordingly, the Mo-induced knockdown of Ephrin B1, Dsh, or PKCδ in dorsal blastomeres of Xenopus embryos causes a mixing of ectoderm and mesoderm cells(Lee et al., 2006; Tanaka et al., 2003).

The activation of effectors of the PCP cascade in response to Ephrin/Eph signaling shows that these pathways are functionally linked in the context of cell sorting and separation (see Figure 18.4). The identification and understanding of mechanisms that mediate the crosstalk between transcription factors, signaling cascades, and cell adhesion will be a challenge for future research.

Another new field of research will be the analysis of separation behavior in nonembryonic systems. During wound healing and organ regeneration, tissue separation has to be developed by the differentiating blastema cells. Stem-cell–derived tissues and organs will have to be tested for their ability to form and recognize tissue boundaries. Finally, it will be important to study whether the loss of separation and repulsion contributes to the formation of metastasizing tumors. The understanding and comparison of the cellular mechanisms that contribute to tissue separation in embryonic and adult tissues will therefore not only be a topic of basic research, but it will also be of interest for the analysis of clinically relevant problems.

SUMMARY

- Tissue separation in the Xenopus gastrula involves the ectoderm, the mesoderm, and the endoderm.
- The endoderm and the mesoderm develop separation and ectoderm repulsion behavior.
- In the mesoderm, the FGF-signaling pathway, the function of the transcription factors Lim1, Mix1 and gsc, the Frizzeld7 receptor, PAPC, and cadherins are required for tissue separation.
- Noncanonical Wnt signaling activates Rho, PKC, and JNK, which regulate morphogenetic cell behaviors, including tissue separation.
- In ectoderm tissue, Ephrin/Eph/Dsh signaling seems to be involved in the repulsion of mesoderm.

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GLOSSARY OF TERMS

Amphibian gastrulation
The infolding and movements of mesoderm cells through the blastopore.

Blastema
The group of embryonic cells from which body structures form during development and regeneration.

Differential adhesion
Specific cell–cell interaction mediated by adhesion molecules.

Ephrin/Eph receptors
Membrane-anchored signaling molecules that interact and mediate repulsion.

Frizzled-mediated signaling
The transduction of extracellular signals trough seven transmembrane receptors of the Frizzled family.

Goosecoid
A homeobox transcription factor expressed in Spemann's organizer.

Lim1
A transcription factor expressed in the dorsal blastopore lip (Spemann's organizer) in Xenopus.

Paraxial protocadherin
An adhesion molecule of the protocadherin protein family that is expressed in the paraxial mesoderm.

Repulsion
A mechanism that specifically prevents the interaction of cells.

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19

ROLE OF THE BASEMENT MEMBRANE IN CELL MIGRATION

KIYOJI NISHIWAKI and YUKIHIKO KUBOTA

RIKEN Center for Developmental Biology, Kobe, Japan

INTRODUCTION

During early embryonic development, blastomeres actively divide with a shortened cell cycle and produce many cells that loosely contact one another. Some of these cells form selective attachments with their neighbors, which often originate from the same or closely related cell lineages, by cadherin-mediated mechanisms (Takeichi, 1995), and they form epithelial sheets that surround the outer surface of organ rudiments. This process, which is called the *mesenchymal–epithelial transition,* is accompanied by the generation of basement membranes, which are extracellular substrates in the form of a matrix of secreted glycoproteins (Shook and Keller, 2003). The formation of basement membranes is mediated mainly by integrin family receptors expressed in the developing epithelia (Bökel and Brown, 2002). The main components of the basement membrane are laminin, type IV collagen, nidogen, and perlecan, but it comprises hundreds of other proteins as well, and abnormalities in the functions of these proteins lead to a range of developmental anomalies and pathogeneses (Kalluri, 2003; Quondamatteo, 2002).

Cell migration is one of the major strategies employed by developing embryos for promoting organ morphogenesis. Cells can migrate individually or collectively in sheets, or they can extend processes while remaining attached to the basement membrane. In this chapter, we describe the molecules that have been found through the genetic analyses of cell and axon migration. A variety of cells and axons migrate along or across the basement membrane during the development of *Caenorhabditis elegans* and *Drosophila melanogaster.* Because invertebrates such as *C. elegans* and Drosophila are essentially devoid of the interstitial extracellular matrix found in vertebrates and often have fewer isoform-encoding genes for basement membrane proteins than do vertebrates (Hutter et al., 2000; Brown et al., 2000), they are invaluable systems for genetically analyzing the function of the basement

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membrane in cell migration. We describe genetic procedures to clarify the logic of the approaches taken in some key experiments. Although the extracellular matrices formed during embryogenesis do not always seem to be clearly discernible by microscopy, the involvement of integrins, dystroglycans, or basement membrane components in the migration process, as shown by genetic analysis, suggests that basement membranes are indeed present. We also introduce here some findings in vertebrate systems to show the evolutionarily conserved mechanisms and the relevance of these findings from model organisms to human genetic diseases. The basement membrane proteins and their receptors discussed in this chapter are listed in Tables 19.1 and 19.2 and Figure 19.1. References for these tables and the figure can be found in the text and online at WormBase (http://www.wormbase.org/), FlyBase (http://flybase.net/), and the National Center for Biotechnology Information protein database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Protein).

On the basis of the analysis of *C. elegans* and Drosophila, there are four major topologically distinct occasions during which cell or axon migration occurs in contact with basement membranes (Figure 19.2). In the first case, ectodermal cells sandwiched between the basement membrane and the plasma membrane of the epidermis migrate or extend processes along the basement

TABLE 19.1 Conservation of Integrins and Dystroglycans Among Species

C. elegans	Drosophila	Mammals
INA-1	Mew (αPS1)	Integrins α3, α6, and α7
PAT-2	If (αPS2)	Integrins α5, α8, αV, and αIIb
None	Scb (αPS3)	None
PAT-3	Mys (βPS)	Integrin β1
DGN-1	Dg	Dystroglycan

If, Inflated; INA, integrin alpha; Mew, multiple edematous wings; Mys, myospheroid; PAT, paralyzed arrest at two-fold stage; PS, position specific; Scb, scab.

TABLE 19.2 Conservation of Basement Membrane Proteins Among Species

C. elegans	Drosophila	Mammals
EPI-1 (αB)	LamA or LanA (α3,5)	Laminins α3, α4, and α5
LAM-3 (αA)	Wb (α1,2)	Laminins α1 and α2
LAM-1	LanB1	Laminins β1 and β2
LAM-2	LanB2	Laminins γ1 and γ2
EMB-9	*Cg52C* product	Type IV collagen α1
LET-2	Viking	Type IV collagen α2
CLE-1	?	Type XVIII collagen
UNC-52	Perlecan	Perlecan
KAL-1	KAL-1 like	Anosmin-1
NID-1	Nidogen	Nidogen-1 and -2
HIM-4 (hemicentin)	?	Hemicentin-1
FBL-1	?	Fibulin-1
GON-1	?	ADAMTS-9 and -20
MIG-17	?	ADAMTS proteins
PPN-1	Papilin	Papilin

CLE, Collagen with endostatin domain; EMB, embryogenesis abnormal; EPI, abnormal epithelia; GON, gonad development abnormal; HIM, high incidence of males; LET, lethal; MIG, migration of cells abnormal; UNC, uncoordinated; Wb, wing blister.

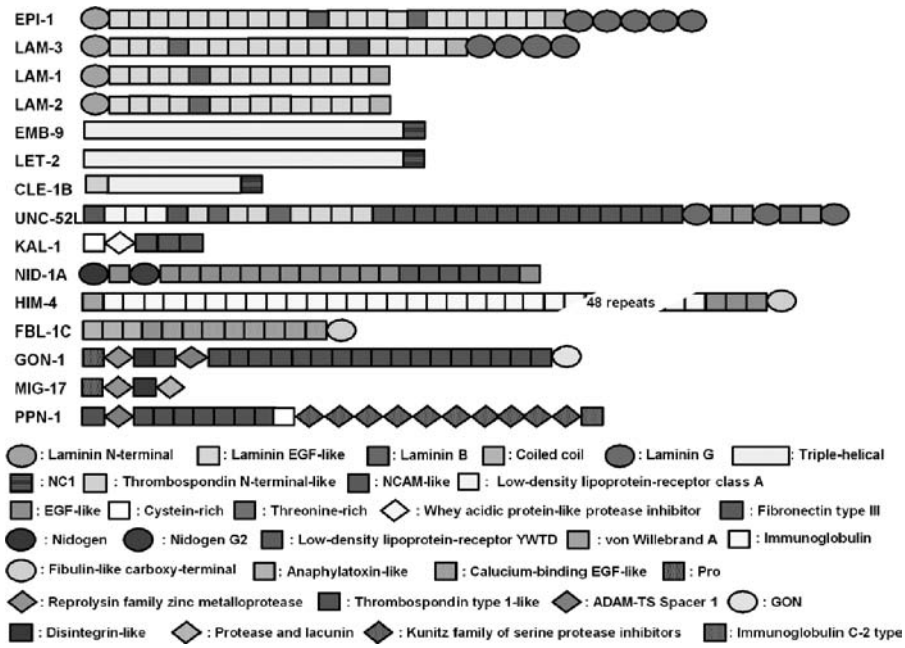

FIGURE 19.1 **Basement membrane molecules.** Representative basement membrane molecules in *C. elegans* are shown in diagrams. N-terminal signal peptides are omitted. The laminin α subunit LAM-3 has only four laminin G domains instead of five in laminin αs found in Drosophila and mammals. HIM-4 (hemicentin) lacks the thrombospondin-type-1–like repeat and the nidogen G2 domain found in human hemicentin-1. NID-1 lacks the thrombospondin-type-1–like domain found in human nidogens. KAL-1 has a putative glycosylphosphatidylinositol anchoring site at the C-terminus that is absent in the other species. (See color insert.)

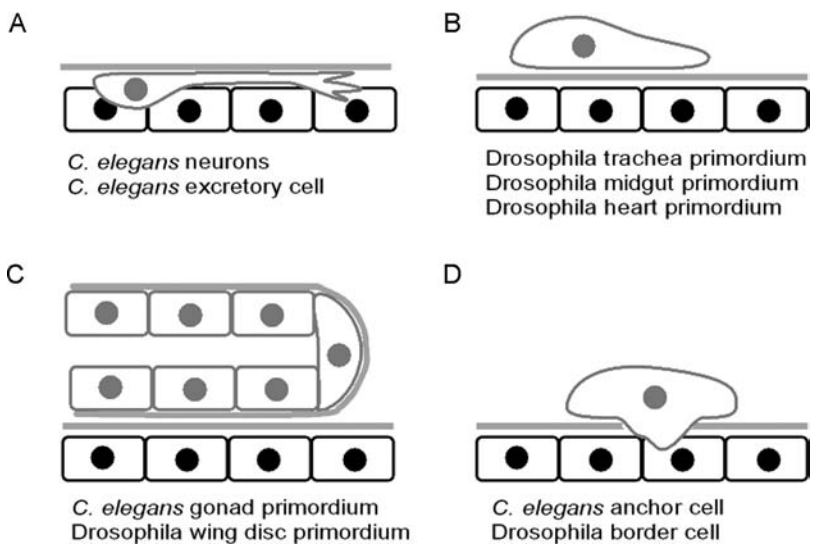

FIGURE 19.2 **The interaction of migratory cells and the basement membrane.** Migratory cells are shown in light gray; cells that constitute the substrate for migration are shown in black; basement membranes are shown in dark gray.

membrane (Figure 19.2, A). This is seen in the migration of neuroblasts and axons and in the extension of excretory canals in *C. elegans*. In the second case, cells migrate over the basement membrane of epithelial sheets (Figure 19.2, B). This is seen in the migration of tracheal branches and midgut and heart primordia in Drosophila. In the third case, epithelial tubes covered with basement membranes migrate over the basement membrane of adjacent epithelial sheets (Figure 19.2, C). This is seen in the migration of *C. elegans* gonad and Drosophila wing disc primordia. In the fourth case, cells degrade the basement membrane and invade neighboring tissues (Figure 19.2, D); this is seen in the migration of *C. elegans* anchor cells and Drosophila border cells.

I. FUNCTION OF EXTRACELLULAR MATRIX RECEPTORS IN CELL MIGRATION

A. Integrins

Integrins are heterodimeric receptors composed of α and β subunits, and they act in various aspects of cell and basement membrane interactions that include the assembly of the basement membrane, cell adhesion to and migration on the basement membrane, and the transduction of signals from the basement membrane to cells (Bökel and Brown, 2002; Hood and Cheresh, 2002; Geiger et al., 2001). There are two β [βPS(Mys) and βv] and five α [αPS1(Mew), αPS2 (If), αPS3(Scb), αPS4, and αPS5] integrin subunits in Drosophila. The βPS subunit is likely to form heterodimers with all five αPS subunits (Brown et al., 2000). One β (PAT-3) and two α (PAT-2 and INA-1) subunits are present in *C. elegans* (Brown, 2000). Therefore, it is the type of the α subunit rather than the β subunit that determines the binding specificity of the αβ heterodimer. Among the α subunits, the functions of αPS1(Mew) and αPS3(Scb) in Drosophila and INA-1 in *C. elegans* are important for migrating cells, whereas those of αPS2(If) and probably PAT-2 are important for cells that assemble the substrate on which migration occurs, as discussed later.

In Drosophila embryos, the outgrowth of the tracheal branch is guided by Branchless (Bnl) fibroblast growth factor (FGF), which is expressed by mesodermal cells (Sutherland et al., 1996). The visceral branch of the developing trachea spreads over the visceral mesoderm and forms the ramified tracheal tree. αPS1 is expressed in the visceral branch, whereas αPS2 is expressed in the visceral mesoderm (Boube et al., 2001). Although mutations in αPS1 and αPS2 cause similar defects in visceral branch migration, the phenotype of *mew* ($\alpha PS1^{-}$) embryos is stronger than that of *if* ($\alpha PS2^{-}$) embryos. The expression of αPS2 in the visceral branch of $\alpha PS1^{-}$ embryos cannot rescue the tracheal branching defects. By contrast, expression of αPS1 in place of αPS2 in the mesoderm substantially rescues the defects, which suggests that either αPS1 or αPS2 integrin can assemble an appropriate substrate for tracheal migration but that αPS1 has a specific function in tracheal cells to promote their migration (Boube et al., 2001). Similar to the case in tracheal migration, integrins are also required for a normal rate of migration of the primordial midgut in Drosophila embryos (Martin-Bermudo et al., 1999). The αPS1 and αPS3 subunits are expressed in migrating primordial midgut cells, whereas αPS2 is expressed in the visceral mesoderm. During midgut migration, αPS1 and αPS3 are partially redundant, and the loss of αPS1 or αPS3 alone results in either a modest delay or no defect in migration, respectively. $\alpha PS1^{-}$ and

αPS3^{-} double-mutant embryos show severe midgut migration delays similar to those seen in the βPS^{-} embryos. Dominant negative Rac and Cdc42 cause a similar defect in midgut migration, which suggests that these small GTPases may function downstream of αPS1βPS and αPS2βPS integrin receptors (Martin-Bermudo et al., 1999).

In *C. elegans ina-1* (α integrin) mutant embryos and larvae, the migration distance of neuroblasts [canal associated neuron (CAN), anterior lateral microtubule cell (ALM), hermaphrodite specific neuron (HSN), and descendants of QR] is shortened, and commissural axon outgrowth is weakly affected (Baum and Garriga, 1997; Poinat et al., 2002). The migration of gonadal distal tip cells (DTCs) is also weakly affected. INA-1 is expressed in all of these cells during their migration (Baum and Garriga, 1997). The function of integrins in DTCs is also suggested by the perturbation of DTC migration by a dominant negative PAT-3 (β integrin; Lee et al., 2001). The other α subunit, PAT-2, is expressed in the body wall muscle cells to form stable focal adhesion to the epidermis (Mackinnon et al., 2002).

B. Dystroglycans

Dystroglycans are transmembrane receptors of the dystrophin-associated glycoprotein complex, which is involved in the pathogenesis of muscular dystrophies (Dalkilic and Kunkel, 2003). Dystroglycans also play a role in basement membrane assembly (Quondamatteo, 2002). The dystroglycan gene in Drosophila, *Dg*, is required for polarity of the epithelial cells and the oocyte (Deng et al., 2003), whereas the corresponding gene in *C. elegans, dgn-1,* is involved in several cell migration events (Johnson et al., 2006). DGN-1 is expressed in various epithelial tissues, including the hypodermal and neural precursors, the gonad primordium, and the excretory cell, but it is not expressed in the muscle. The excretory canals, which are tubular arms of the excretory cell, are missing or shortened, and commissures of DA/DB motor neurons extend on the wrong side in *dgn-1* null mutants. Although the gonads rupture in *dgn-1* homozygotes, the DTCs in *dgn-1*/+ heterozygotes show misdirected migration at a low frequency. Thus, *dgn-1* probably has a cell-autonomous function in migrating cells or axons but not in muscle cells (Johnson et al., 2006).

Vertebrate dystroglycans also function in morphogenetic events in nonmuscle cells. Blocking the binding of α-dystroglycan to laminin using antibodies against α-dystroglycan or laminin perturbs the branching morphogenesis of kidney, salivary gland, and lung epithelia (Durbeej et al., 1995; 2001). The brain-specific knockout of mouse dystroglycan causes a disarray of cerebral cortical layering, a fusion of cerebral hemisphere and cerebellar folia, and an aberrant migration of granule cells (Moore et al., 2002). The pial surface basement membrane of the knockout mouse becomes discontinuous as a result of the weakened affinity to laminin (Moore et al., 2002).

II. FUNCTION OF BASEMENT MEMBRANE PROTEINS IN CELL MIGRATION

A. Laminins

Laminins are major players among the basement membrane proteins that are involved in interacting with membrane receptors such as integrins and dystroglycans (Miner and Yurchenco, 2004). They are heterotrimeric basement

membrane proteins that consist of α, β, and γ chains. Five α, four β, and three γ laminin chains are expressed in mammals, and these can assemble into 15 different types of αβγ trimers. By contrast, two α, one β, and one γ chains form only two types of αβγ trimers in *C. elegans* and Drosophila (Miner and Yurchenco, 2004). The *C. elegans* LAM-3 (αA) and the Drosophila Wb (α1,2) are similar to mammalian α1 and α2. The *C. elegans* EPI-1 (αB) and the Drosophila LamA (α3,5) are similar to mammalian α3, α4, and α5.

In *C. elegans*, strong alleles or double-stranded RNA-mediated gene interference (RNAi) of *epi-1* (αB) and *lam-3* (αA) have embryonic, larval lethal, or sterile phenotypes. However, RNAi of *lam-1* (β), *lam-2* (γ), or *epi-1* plus *lam-3* results in much stronger embryonic lethal phenotypes, which suggests some functional redundancy in the two α subunit genes (Huang et al., 2003a; Kao et al., 2006). Although both *epi-1* and *lam-3* mutations result in the disruption of basement membranes and the abnormal adhesion of tissues, *epi-1* also exhibits defects in neuroblast migration, in axon migration and fasciculation, and in the extension of excretory canals (Huang et al., 2003a; Johnson et al., 2006).

The basement membranes of the epidermis, muscles, and gonads contain only EPI-1. Because neurons and excretory canals are located between the epidermal basement membrane and the plasma membrane of the epidermis, EPI-1 probably plays important roles in patterning the nervous and excretory systems. However, LAM-3 specifically accumulates in the regions of the basement membrane that are associated with nerve tracts, which suggests that specific cell surface receptor(s) for LAM-3 may be present in neurons (Huang et al., 2003a). *epi-1* mutants show widespread neuronal defects that are mostly shared with mutations in *mig-2*, *unc-73,* and *ina-1* (Forrester and Garriga, 1997). UNC-73 is a guanine nucleotide exchange factor similar to Drosophila's Trio (Steven et al., 1998), and MIG-2 is a Rac-related protein that acts downstream of UNC-73 (Zipkin et al., 1997). INA-1 is one of the two integrin α subunits that form a heterodimeric receptor with the β subunit PAT-3, and it probably binds laminins (Baum and Garriga, 1997). Thus, the binding of EPI-1 to INA-1/PAT-3 may induce an intracellular signal, which could be mediated by UNC-73 and MIG-2 to promote cell and axon migration. Mutations in *lam-1* (laminin β) also result in the disruption of the basement membranes, and weak alleles exhibit gonadal DTC migration defects (Kao et al., 2006).

The disruption of the gonadal basement membrane and the defective extension of excretory canals in *epi-1* mutants are phenotypically very similar to those seen in *dgn-1* mutants, which suggests the possibility that DGN-1 (dystroglycan) acts as a receptor for EPI-1. EPI-1 does, however, localize to the gonadal basement membrane in *dgn-1* mutants (Johnson et al., 2006). This result raises the possibility that DGN-1 is not a direct receptor for EPI-1 that leads to assembly of the basement membrane; rather, it could function in transducing signals from the EPI-1–containing basement membrane or act in the organization of the basement membrane while interacting indirectly with EPI-1.

In Drosophila, LamA (α3,5) is distributed widely in basement membranes during organogenesis in late embryos (Fessler and Fessler, 1989; Kusche-Gullberg et al., 1992; Montell and Goodman, 1989). Mutations in weak alleles of *LamA* (α3,5) cause a series of migration defects during embryogenesis and pupal stages (Yarnitzky and Volk, 1995). During embryonic heart formation, the pericardial cells cannot form a coherent line as do those in the wild type; rather, they dissociate and migrate randomly. The rows of cardioblasts

become twisted and discontinuous. *LamA* mutant embryos also have gaps in the dorsal trunk of the trachea (Stark et al., 1997). Similar defects have also been reported in *wb* (α1,2) mutants (Martin et al., 1999) and in αPS3^{-} and βPS^{-} embryos (Stark et al., 1997; Boube et al., 2001). The phenotypic similarity among *LamA*, *wb*, αPS3^{-} and βPS^{-} mutants suggests that LamA and Wb are ligands for the αPS3βPS integrin and that they function in various aspects of movement and morphogenesis in Drosophila embryogenesis. Recently, the migration of wing disc primordia over the tracheal branch was analyzed. Wb accumulates on the wing primordia, whereas LamA accumulates on the tracheal branch. Genetic analysis revealed that Wb, αPS2, and βPS—but not LamA—are required for this migration (Inoue and Hayashi, 2007), which suggests that Wb acts as a ligand for the αPS2βPS integrin in wing disc migration. In addition to these embryonic cell migration defects, *LamA* mutants also display strong pathfinding defects in ocellar pioneer axons in the pupal stage (Garcia-Alonso et al., 1996). The ocellar pioneer axons normally fasciculate with one another to form bundles and project into the brain; alternatively, in *LamA* mutants, they migrate a short distance along the epidermis; they often stall or sometimes fasciculate with mechanosensory axons, but they never reach the brain.

Mutations in the human laminin chains can lead to junctional epidermolysis bullosa, which is characterized by blister formation within dermal–epidermal basement membranes (McGowan and Marinkovich, 2000). Although not directly associated with cell migration, this phenotype is reminiscent of the blister formation in fly wings found in *wb* and *LamA* mutants (Henchcliffe et al., 1993; Martin et al., 1999).

B. Basement Membrane Collagens

Six type IV collagen genes have been identified in mammals (Hudson et al., 1993). A triple-helical trimer composed of two α1 and one α2 chains is the predominant form found in the mammalian basement membrane. *C. elegans* and Drosophila have single α1 and α2 subunit genes; mutations in these lead to embryonic lethality (Brown et al., 2000; Kramer, 2005). The Drosophila type IV collagen may play a role in the invasion of border cells to form the apical cap, as discussed later. The function of type IV collagen in cell and axon migration in *C. elegans* remains to be determined.

Type XVIII collagen has large N- and C-terminal globular domains and a short collagenous domain with multiple interruptions (Oh et al., 1994; Rehn et al., 1994). The C-terminal 20-kDa fragment, called *endostatin* (ES), as well as the 40-kDa noncollagenous (NC1) fragment that contains the ES domain, can be physiologically produced by proteolytic processing of type XVIII collagen in mammals (O'Reilly et al., 1997; Yamaguchi et al., 1999; Sasaki et al., 1998). CLE-1 (type XVIII collagen) is expressed in the basement membranes of various tissues at low levels but accumulates to a high degree in the nervous system in *C. elegans* (Ackley et al., 2001). A *cle-1* mutation that deletes the C-terminal NC1 domain (a region of about 40 kDa that contains the 20-kDa ES domain) was isolated (Ackley et al., 2001). This mutant exhibits various cell and axon migration defects: a shortened migration of neurons ALM, anterior ventral microtubule cell (AVM), and HSN; a misdirected migration of commissural axons; and the premature dorsal turning of the gonadal DTCs. Interestingly, the expression of the NC1 domain under the control of a touch

neuron-specific promoter rescues neuronal migration defects but not the gonadal migration defects of *cle-1* mutants. The expression of the ES domain (but not, however, of the entire NC1 domain) in wild-type animals causes *cle-1* mutant-like migration phenotypes. Furthermore, the expression of the ES domain in *cle-1* animals has no effect, which suggests that the NC1 domain has promigratory activity that might be negatively regulated by ES (Ackley et al., 2001). Similar promigratory and antimigratory activities for NC1 and ES, respectively, have also been reported in the migration and morphogenesis of cultured human endothelial cells (Kuo et al., 2001). Although ES exists as a monomer, the NC1 fragment exists as a trimer that contains three ES domains; this suggests that the trimerization of ES can convert its antimigratory activity into a promigratory one (Ackley et al., 2001; Kuo et al., 2001). The antiangiogenic activity of ES has been the focus of cancer therapy research as a means of inhibiting the sprouting of new microvessels, which would thus lead to tumor cell death (Kerbel and Folkman, 2002).

C. Proteoglycans

The ventral-to-dorsal migration of gonadal DTCs is regulated by the guidance molecule UNC-6 (netrin) and its receptors, UNC-5 and UNC-40, which are expressed in DTCs in *C. elegans* (Hedgecock et al., 1990). Genetic-enhancer screening of DTC migration defects for the weak *unc-5* allele identified mutations in the *unc-52* gene, which encodes perlecan, a basement membrane heparan sulfate proteoglycan (Merz et al., 2003). Although *unc-52* null mutants are lethal, these enhancer *unc-52* mutations, which have missense mutations in the neural cell adhesion molecule (NCAM)-like immunoglobulin repeats at the middle of the molecule, are viable, and they have no DTC migration defects by themselves. Heparan sulfate proteoglycans are known to regulate the tissue distribution of extracellular signaling molecules (Bernfield et al., 1999). The introduction of mutations in the extracellular signaling molecules *unc-129* tranforming growth factor (TGF)β, *dbl-1* (TGFβ), *egl-20* (Wnt), and *egl-17* (FGF) in *unc-5* and *unc-52* double mutants partially suppresses the enhanced DTC migration defects caused by *unc-52* (Merz et al., 2003). Thus, the *unc-52* mutations probably cause these signaling molecules to be inappropriately active so that they induce DTC migration defects, which is consistent with the possible function of UNC-52 (perlecan) in binding and sequestering extracellular signaling molecules. The UNC-52 protein accumulates at the muscle–epidermal interface and in the pharyngeal basement membrane but not in the gonadal basement membrane or the body wall basement membrane facing the body cavity on which DTCs migrate (Merz et al., 2003). Therefore, it is possible that UNC-52 in basement membranes that do not contact DTCs act in the signaling for DTC migration. Alternatively, although the levels of UNC-52 are too low to be detected in the gonadal or body wall basement membranes, the amounts are sufficient to support normal DTC migration. The function of perlecan is not known in Drosophila. Null mutations in the human perlecan gene result in dyssegmental dysplasias of the Silverman–Handmaker type, which are characterized by skeletal dysplasias with anisospondyly and micromelia (Arikawa-Hirasawa et al., 2001). Hypomorphic mutations cause Schwartz–Jampel syndrome, which is characterized by skeletal dysplasias with myotonia (Arikawa-Hirasawa et al., 2002). Although it is not clear whether the function of perlecan is required for cell

migration in mammals, further cell biologic analysis using mouse models should address this issue.

There are many reports concerning genes that are required for the synthesis or modification of heparan sulfate, which forms side chains for both membrane-bound and secreted proteoglycans, and mutations in these genes affect axon pathfinding in various animal models (Lee and Chien, 2004; Häcker et al., 2005). In addition, the functions of membrane-bound proteoglycans, syndecans, and glypicans in axon migration have been reported (Häcker et al., 2005). Although the involvement of guidance molecules such as FGF, Slit, Wnt, Hh, TGFβ, and bone morphogenetic protein has been analyzed in these studies, the functions of the basement membrane are still generally unknown. However, it is possible that proteoglycans localized to the basement membrane or membrane-bound proteoglycans, which may interact with the underlying basement membrane, act in the regulation of axon migration.

D. Kallmann Syndrome Protein (Anosmin-1)

Kallmann syndrome is a hereditary disease that is characterized by hypogonadotropic hypogonadism and anosmia, which is the inability to smell (Kallmann et al., 1944). The affected patients exhibit abnormalities in the axon guidance of olfactory neurons and of the neurons that secrete gonadotropin-releasing hormone (Schwanzel-Fukuda et al., 1989). Anosmin-1, which is the product of the Kallmann syndrome gene (*Kal-1*), associates with basement membranes and the interstitial matrix of the embryonic brain (Hardelin et al., 1999; Soussi-Yanicostas et al., 2002). The *C. elegans* ortholog of the human protein KAL-1 has a whey acidic protein domain (a WAP-like protease inhibitor domain) and three fibronectin type III domains, and it is expressed in ventral neuroblasts in embryos and in head and tail ganglia in larvae (Bülow et al., 2002; Rugarli et al., 2002). Loss-of-function mutations or overexpression of the *kal-1* gene causes similar defects in epidermal ventral closure and neurite outgrowth during embryogenesis, which suggests that the level of KAL-1 is strictly controlled during development (Bülow et al., 2002; Rugarli et al., 2002). The overexpression of KAL-1 in the AIY neuron, in which KAL-1 is normally expressed, induces a highly penetrant axon-branching defect. Using ethylmethane sulfonate mutagenesis, mutations that suppress the defective branching of *kal-1*–overexpressing animals were isolated. Among the mutations were those in the gene *hst-6,* which encodes heparan-6-*O*-sulfotransferase, an enzyme that is required for the sulfate modification of heparan sulfate polysaccharide chains of proteoglycans (Bülow et al., 2002). The wild-type KAL-1 protein shows heparin-dependent cell adhesion activity, but a mutant KAL-1 protein that has an amino acid substitution that corresponds with a human Kallmann syndrome mutation does not. Thus, KAL-1 requires heparan sulfate proteoglycans for its activity in cell and axon migration (Bülow et al., 2002). Human KAL-1 also binds heparan sulfate (Hu et al., 2004). Because KAL-1 acts in a cell-autonomous manner in axon outgrowth, it may localize to the surface of axons or to basement membranes immediately underlying axons, and it may contribute to the appropriate adhesion between axons and basement membranes to suppress inappropriate branching. Thus, it is likely that heparan sulfate proteoglycans bind KAL-1 as a cofactor and that they play an essential role in axonogenesis.

E. Nidogen Interacts with a Netrin Receptor

Although nidogen is one of the major components of the basement membrane, it is not essential for basement membrane assembly or *C. elegans* viability (Kang and Kramer, 2000; Kim and Wadsworth, 2000). There are two nidogen genes in mammals and only one in *C. elegans, nid-1*. Although *nid-1* mutants have no gross abnormalities in morphogenesis or behavior, abnormalities are detected in the positioning of neurites (Kim and Wadsworth, 2000). These include effects seen in the bilateral PVQ axons in the ventral cord, in which the left axon crosses over the ventral midline to the right fascicle. The motor axons that normally run in the right fascicle of the ventral cord also populate the left fascicle in *nid-1* mutants. Additionally, dorsal–sublateral nerves are shifted to the dorsal midline in *nid-1* mutants. One such neuron, SDQR, which has a cell body that is at the ventral–sublateral position, extends an axon dorsally; this axon is reoriented anteriorly at the dorsal–sublateral position (where NID-1 accumulates) in the wild type, but the reorientation occurs at the dorsal midline in *nid-1* mutants. In *unc-40* single or *unc-40* and *nid-1* double mutants, however, the SDQR axons are often reoriented at the dorsal–sublateral position, as they are in the wild type. The UNC-40 protein is an ortholog of mammalian deleted in colorectal cancer (DCC), a receptor for the dorsal–ventral axon guidance molecule UNC-6 (netrin) (Chan et al., 1996). These results suggest that NID-1 interacts with UNC-40 to reorient the SDQR axons. NID-1 might negatively regulate the UNC-40–mediated dorsal guidance so that the SDQR axon can respond to the anterior guidance activity that is latent when UNC-40 is active.

F. Basement Membrane Regulation in Cell Invasion

Cancer cells invade tissues by proteolytically removing basement membranes. Such cell invasion is also observed in normal development, such as in trophoblast implantation and gastrulation. In *C. elegans,* gonadal anchor cells invade the vulval epithelium across the basement membranes that separate both tissues and connect the developing uterus and vulva (Sherwood and Sternberg, 2003). Laser ablation of the anchor cell before invasion results in an everted vulval phenotype (Kimble, 1981; Seydoux et al., 1993). One of the everted vulva mutants, *fos-1,* is defective in anchor cell invasion. *fos-1* encodes two alternatively spliced orthologs of the fos bZIP transcription factor family, which is found in vertebrates (Sherwood et al., 2005). One of the two isoforms, FOS-1A, which is expressed strongly in the anchor cell, rescues *fos-1* mutant defects. Screening of known genes expressed in the anchor cell identified three genes—*zmp-1* (GPI-anchored membrane-type metalloprotease), *cdh-3* (Fat-like protocadherin), and *him-4* (hemicentin, an extracellular matrix protein) (Vogel and Hedgecock, 2001)—for which expression becomes undetectable or weak in *fos-1* mutants. These genes could be the targets of FOS-1A, and they may act together to promote invasion. Interestingly, HIM-4 (hemicentin) begins to accumulate in the basement membrane under the anchor cell, and this accumulation continues to increase until the time of invasion. The deposited HIM-4 is cleared, leaving large aggregates surrounding the invasion site when the anchor cell begins its invasion. It is possible that hemicentin promotes basement membrane removal, either through the structural modification of the basement membrane or by providing increased adhesion between the anchor cell and the basement membrane (Sherwood et al., 2005).

Such transient accumulation followed by the removal of basement membrane components is also observed in Drosophila oogenesis (Medioni and Noselli, 2005). The polar cell, accompanied by outer border cells (BC cluster), delaminates from the anterior follicular epithelium and invaginates into the egg chamber (Montell, 2003). The preinvasive polar cells undergo an unusual apical capping with major basement membrane proteins, including type IV collagen α1 and α2 chains, LamA, and perlecan (Medioni and Noselli, 2005). The apical cap is cleared when the BC cluster starts to migrate. Interestingly, the components of the apical cap are transported from the basement membrane surrounding the egg chamber by Drab5-dependent transcytosis. The outer border cells are essential for shedding the apical cap. Although the apical capping is suggested to play a role in blocking the migration of immature clusters (Medioni and Noselli, 2005), it is also possible that the transient capping is required for promoting migration, as suggested by the case of *C. elegans* anchor cells.

III. FUNCTION OF EXTRACELLULAR PROTEASES IN CELL MIGRATION

A. ADAMTS Proteases

Members of the ADAMTS (a disintegrin and metalloprotease with thrombospondin motifs) family of secreted proteins play important roles in animal development and pathogenesis (Porter et al., 2005). ADAMTS proteases are characterized by structural features: an N-terminal signal peptide, a prodomain, a metalloprotease (MP) domain, a disintegrin (DI) domain, a variable number of thrombospondin type I (TS) motifs, and some other ancillary domains near the C terminus. In *C. elegans*, two secreted ADAMTS proteins, GON-1 and MIG-17, act from outside the gonad to control the migration of gonadal leader cells that promote gonad morphogenesis (Blelloch and Kimble, 1999; Nishiwaki et al., 2000).

Development of the *C. elegans* hermaphrodite gonad is shown schematically in Figure 19.3. The gonad primordium lies at the center of the body over the ventral body wall muscle at the first larval (L1) stage. The primordium elongates arms both anteriorly and posteriorly during the L2 and early L3 stages. The gonad arms turn dorsally during the mid-L3 stage and migrate across the lateral hypodermis. They turn again over the dorsal muscle around the time of the L3 molt and subsequently migrate toward each other along the dorsal muscle. The migration of gonad arms is led by DTCs, which are located at the anterior and posterior ends of the gonad (Kimble and White, 1981; Hedgecock et al., 1987).

Although gonad primordia develop normally in *gon-1* mutants, DTC movement is blocked, and the gonads develop as disorganized masses of somatic and germline tissues (Figure 19.4, *A*) (Blelloch and Kimble, 1999; Blelloch et al., 1999). GON-1 is most similar to mammalian ADAMTS-9 and -20 (Somerville et al., 2003). GON-1 is expressed in both body wall muscles and DTCs during gonad development (Blelloch and Kimble, 1999). When GON-1 is expressed only in muscle cells in *gon-1* mutants, the gonad undergoes pronounced swelling, and DTC migration defects are not rescued (Figure 19.4, *B*). Normal migration is recovered in *gon-1* mutants when GON-1 is expressed in DTCs rather than in muscle cells, although the gonad arms

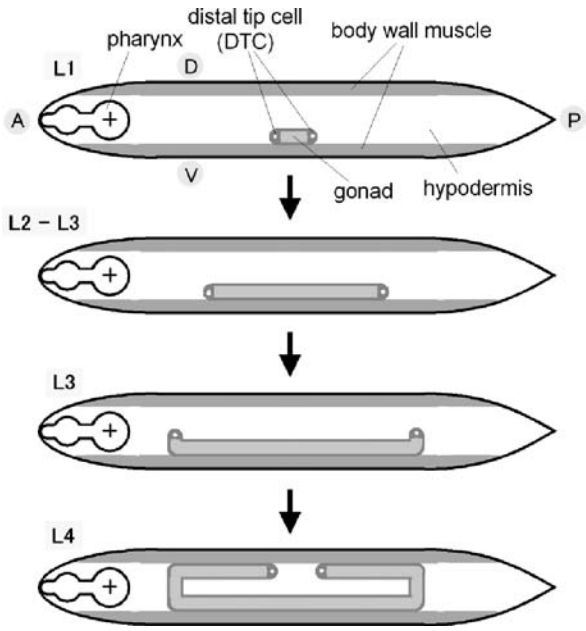

FIGURE 19.3 **Development of the *C. elegans* gonad.** A, P, D, and V correspond with anterior, posterior, dorsal, and ventral, respectively. L1, L2, L3, and L4 represent first, second, third, and fourth larval stages, respectively. The gonadal basement membrane is shown in brown, and distal tip cells are shown in red. The basement membranes of the body wall muscle and hypodermis are not shown. (See color insert.)

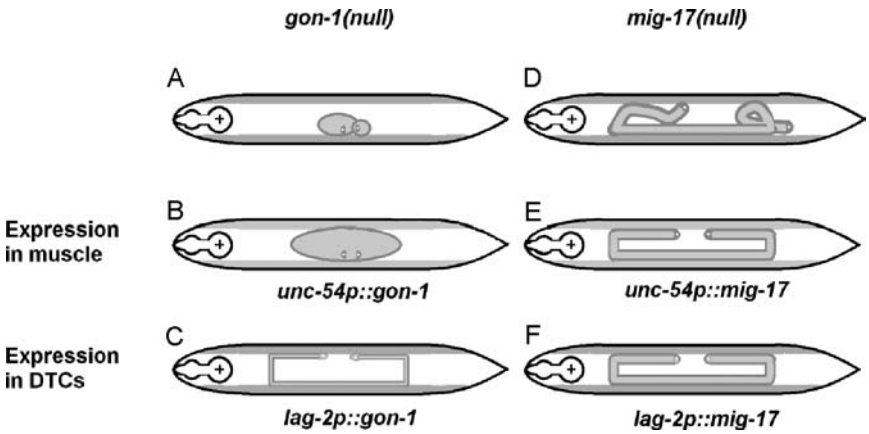

FIGURE 19.4 **Rescue experiments by tissue-specific expression of GON-1 and MIG-17.** The *unc-54* promoter and *lag-2* promoter express downstream genes in body wall muscle cells and distal tip cells, respectively. The tissues expressing GON-1 or MIG-17 are shown in green. (See color insert.)

are thinner than normal (Figure 19.4, C). Therefore, GON-1 that is secreted from the body wall muscle cells differs in function from that which is secreted from the DTCs. The former seems to be required for the expansion of the gonad, whereas the latter allows DTCs to migrate (Blelloch and Kimble, 1999).

In *mig-17* mutants, the initial migration of DTCs on the ventral body wall muscle is normal, even in the *mig-17* null mutant. However, DTCs do deviate from the normal pathway after the first turn, and they meander over the body

wall (Figure 19.4, D). Therefore, *mig-17* is not required for the act of migration itself but rather for directing DTC migration via appropriate interactions between the basement membrane of the gonad and that of the body wall. Although lacking TS motifs, MIG-17 apparently belongs to the ADAMTS family based on the significant homology between its MP, DI, and C-terminal protease and lacunin domains and those found in ADAMTS proteins (Nishiwaki et al., 2000; Nishiwaki, unpublished data). MIG-17 is secreted from the body wall muscle cells, and it accumulates in the gonadal basement membrane after the first turn of the DTCs. These expression kinetics coincide with the onset of DTC migration abnormalities in *mig-17* mutants. As expected, *mig-17* expression under a muscle-specific promoter rescues *mig-17* DTC migration defects (Figure 19.4, E). In addition, *mig-17* expression under the control of a DTC-specific promoter also rescues the mutant phenotypes (Figure 19.4, F), which suggests that MIG-17 activity in the basement membrane on the surface of DTCs is important for normal gonadogenesis (Nishiwaki et al., 2000). Therefore, these two ADAMTS proteases, GON-1 and MIG-17, act together to control gonad morphogenesis in *C. elegans*.

B. Mutations in Fibulin-1 Suppress Both *mig*-17 and *gon*-1 Mutations

The isolation and analysis of suppressor mutations are frequently used approaches in genetic analysis to identify interacting molecules. Suppressor screening was also used in the analysis of the KAL-1 function in axon migration, as discussed previously. It was found that mutations in the *C. elegans* homolog of fibulin-1, FBL-1, can act as suppressors for the gonadal defects in *gon-1* and *mig-17* animals (Hesselson et al., 2004; Kubota et al., 2004). Fibulin-1 is an extracellular matrix protein that is found in mammals and that is known to reside in the basement membranes, extracellular elastic fibers, and blood plasma (Timpl et al., 2003). Because a deletion mutant of the *fbl-1* gene can partially suppress a null allele of *gon-1* and vice versa, it has been suggested that FBL-1 and GON-1 have antagonistic roles in gonad development (Hesselson et al., 2004). Alternatively, a deletion mutant of *fbl-1* cannot suppress a *mig-17* null mutant, although specific amino-acid substitutions in the third epidermal growth factor-like motif of FBL-1 strongly suppress *mig-17* mutations (Kubota et al., 2004). Of the two spliced isoforms, FBL-1C and FBL-1D, FBL-1C is responsible for the suppression. FBL-1C is secreted from the gut cells, and it localizes to the gonadal basement membrane in a MIG-17–dependent manner. Because these suppressor *fbl-1* mutations are dominant gain-of-function mutations, they could mimic the molecular events normally elicited by MIG-17–dependent proteolysis. Interestingly, these suppressor *fbl-1* mutations also strongly suppress a weak *gon-1* allele (Kubota and Nishiwaki, unpublished data). It might be possible that GON-1 and MIG-17 function in a sequential manner to control gonad development and that FBL-1 is required for both of these steps. Although it is possible that FBL-1 might be the substrate of MIG-17 and/or GON-1, no evidence of this relationship has been obtained (Hesselson et al., 2004; Kubota et al., 2004).

Mutations in human ADAMTS proteins cause various hereditary diseases that are related to disorders in extracellular matrices (Porter et al., 2005). For example, ADAMTS-2 is a procollagen propeptidase, and mutations in this gene result in Ehlers–Danlos syndrome, a connective tissue disorder characterized by extreme skin fragility (Colige et al., 2004). ADAMTS-13 is

a von Willebrand factor–cleaving enzyme that is required for blood clotting; mutations result in thrombotic thrombocytopenic purpura (Levy et al., 2001). Defects in ADAMTS-10, although its substrate is unknown, cause the Weill–Marchesani syndrome, which is characterized by short stature, brachydactyly, joint stiffness, and eye anomalies (Dagoneau et al., 2004). In addition, mutations in mouse ADAMTS-20, which is similar to GON-1, result in a belted white-spotting phenotype, which may be the result of the defective migration of melanoblasts from the neural crest (Rao et al., 2003). Therefore, ADAMTS-20 may function in cell migration, as does GON-1.

Fibulins are also known to play important roles in pathogenesis (Chu and Tsuda, 2004). A mutation in the D isoform of fibulin-1 is associated with a complex type of synpolydactyly (Debeer et al., 2002). Fibulin-3, which is also called EFEMP1 (epidermal growth factor–containing fibrillin-like protein 1), and fibulin-5 are implicated in retinal dystrophies (Stone et al., 1999; 2004). Mutations in fibulin-4 and fibulin-5 cause the cutis laxa syndrome, a connective tissue disorder characterized by loose skin and variable systemic manifestations (Loeys et al., 2002; Hucthagowder et al., 2006). Involvement of fibulin-5 in the development of elastic fibers has been shown by knockout mice, which develop marked elastinopathy with vascular abnormalities, severe emphysema, and loose skin (cutis laxa; Nakamura et al., 2002; Yanagisawa et al., 2002). Although the interaction between fibulins and ADAMTSs is mostly unexplored in vertebrates, mammalian fibulin-1 was recently reported to act as a cofactor to enhance the aggrecanase activity of ADAMTS-1 *in vitro* (Lee et al., 2005). This is intriguing, considering the proposed interaction between fibulin-1 and MIG-17 or GON-1 in *C. elegans*.

C. Noncatalytic ADAM and ADAMTS–Like Proteins

ADAM (a disintegrin and metalloprotease) family proteins are transmembrane proteins, and more than 30 members were found in mammals. Interestingly, about half of the members are likely to be catalytically inactive because of alterations in their active sites (Huovila et al., 2005; White, 2003). *C. elegans* UNC-71 is one such noncatalytic ADAM that is required for the axon guidance of type D neurons and the migration of sex myoblasts (Huang et al., 2003b). Interestingly, UNC-71 acts non–cell-autonomously to regulate these axon and cell migrations: the function of UNC-71 is required in hypodermis and in some neurons other than D neurons. Although it has been proposed that ADAMs with inactive metalloprotease domains could function as inhibitors of active ADAMs (Pan and Rubin, 1997), the double-mutant combinations of *unc-71* along with the potentially active ADAMs *adm-2*, *adm-4*, and *sup-17* do not reveal the suppression of the axon guidance defects in *unc-71* mutants; this suggests that UNC-71 is unlikely to act through other ADAMs. However, *unc-71* mutants do exhibit strong synergism with integrin mutants *ina-1* (α integrin) and *pat-3* (β integrin) for axon fasciculation and also with *unc-6* (netrin) and *unc-5* (netrin receptor) mutants for the dorsal axon guidance of D neurons (Huang et al., 2003b). Integrins and UNC-5 receptors are expressed in D neurons, and UNC-6 may be associated with the basement membrane. Although actual mechanisms for the synergism are not clear, such synergistic effects suggest the possibility that UNC-71 alters the distribution or conformation of ligands in the basement membrane so that D neurons are guided appropriately.

Drosophila papilin is an ADAMTS-like secreted protein with Kunitz protease inhibitor-like repeats, but it lacks a metalloprotease domain (Kramerova et al., 2000). Although RNAi of *papilin* results in embryonic lethality, ectopic expression causes defective elongation of malpighian tubules and disordered arrangements of muscles. Papilin noncompetitively inhibits a chick procollagen N-peptidase (ADAMTS) *in vitro*. Papilin might act in cell arrangement and migration by inhibiting ADAMTS proteases during Drosophila development.

IV. PERSPECTIVES

Basement membranes are formed at the basal surface of polarized epithelia, and they serve as an extracellular environment that has important roles in morphogenetic processes. Cells secrete various adhesive and signaling molecules into the basement membrane to form the environment that is required for regulating the morphogenetic movement of cells. The extracellular environment can be modified or remodeled by, for example, proteases secreted or exposed by cells during development. The interaction between cells and the extracellular environment is a dynamic process with molecular mechanisms that remain poorly understood. Because molecules in the extracellular environment participate in supermolecular assemblies, it is often difficult to apply conventional biochemical approaches to analyze their properties. Genetic analyses using the simple model organisms *C. elegans* and Drosophila offer a rigorous way to examine the function of extracellular molecules *in vivo*, and they should be one of the key approaches to understanding the molecular mechanisms of the interaction between migratory cells and their extracellular environments. The components of the basement membrane and their receptors are well conserved from vertebrates to invertebrates, and the functional conservation has also been revealed in several examples, as discussed in this chapter. Therefore, the analysis of molecular pathways in these invertebrate systems should be particularly helpful for understanding vertebrate development and human diseases.

SUMMARY

- Interactions between migrating cells and the basement membrane can be classified into four topologically distinct cases: (1) migrating cells are between the basement membrane and the plasma membrane of the epidermis; (2) individual cells migrate over the basement membrane of the epithelium; (3) epithelial tubes with basement membranes migrate over the basement membrane of the adjacent epithelium; and (4) cells invade neighboring tissues by degrading the basement membrane.
- Integrins and dystroglycans are major receptors for basement membrane proteins, and they play important roles in cell migration. Five integrin α and two β subunits are present in Drosophila. Two integrin α and a single β subunits are found in *C. elegans*. The α subunits—INA-1 in *C. elegans* and αPS1 and αPS3 in Drosophila—are often expressed in migrating cells, whereas PAT-2 in *C. elegans* and αPS2 in Drosophila are expressed in cells that assemble the substrate for migration.

- Laminins act as ligands for integrins and dystroglycans. Two types of laminin α subunits and single β and γ subunits are present in both *C. elegans* and Drosophila. The two laminin trimers seem to have partially overlapping functions.
- The NC1 and ES fragments of type XVIII collagen have promoting and inhibitory functions, respectively, in cell migration.
- Heparan sulfate proteoglycans act in cell migration through the spatial and temporal regulation of extracellular signaling molecules. Kallmann syndrome protein anosmin-1 binds to heparan sulfate proteoglycans and acts in axon guidance.
- Nidogen interacts with the UNC-40 netrin receptor and affects axon pathfinding in *C. elegans*.
- The ADAMTS family proteases GON-1 and MIG-17 interact with FBL-1 (fibulin-1) and control DTC migration in *C. elegans*. FBL-1 accumulates at the gonadal basement membrane in a MIG-17–dependent manner. Some ADAM and ADAMTS–like proteins that function in migration or morphogenesis are catalytically inactive.

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GLOSSARY OF TERMS

Brachydactyly
Shortness of the fingers and toes.

Dyssegmental dysplasias
Lethal forms of neonatal short-limbed dwarfism. Dyssegmental dysplasia was named by Handmaker et al. (1977) for a marked difference in the size and shape of the vertebral bodies (anisospondyly), which was attributed to segmentation errors.

Emphysema
A lung condition featuring an abnormal accumulation of air in the lung's many tiny air sacs (alveoli). As air continues to collect in these sacs, they become enlarged, and they may break or become damaged and form scar tissue.

Micromelia
Abnormally small arms or legs.

Synpolydactyly
The cutaneous or bony fusion of fingers and toes often associated with additional digital elements within the web.

Thrombotic thrombocytopenic purpura
A blood disorder characterized by blood clots that form in small blood vessels throughout the body that can cause serious medical problems by restricting blood flow to organs such as the brain, kidneys, and heart.

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20
EPITHELIAL MORPHOGENESIS

RONIT WILK[1,2] and HOWARD D. LIPSHITZ[1,2]

[1]*Department of Medical Genetics & Microbiology, University of Toronto;*
[2]*Program in Developmental and Stem Cell Biology, Research Institute, The Hospital for Sick Children, Toronto, Ontario, Canada*

INTRODUCTION

During embryogenesis, simple epithelial sheets are converted into functional three-dimensional tissues and organs; this process is referred to as *epithelial morphogenesis*. The process is regulated in space and time by signaling molecules and transcription factors, which orchestrate coordinated changes in cell shape, cell–cell and cell–extracellular matrix (ECM) contact, cell movement, cell division, and programmed cell death.

Our current understanding of these processes has derived from the study of cultured vertebrate cells and, more recently, from molecular genetic and microscopic analyses (particularly live imaging) of embryos of model organisms such as the insect *Drosophila melanogaster,* the nematode worm *Caenorhabditis elegans,* the zebrafish *Danio rerio,* and the mouse *Mus musculus*.

This chapter begins by describing the molecular architecture of epithelial cells in vertebrates and invertebrates. We then focus on specific types of epithelial morphogenesis and illustrate how the combined use of genetically accessible animal models and sophisticated imaging techniques has led to the in-depth understanding of the molecular and cellular events that underlie these processes. Finally, we discuss several examples of diseases that are caused by defects in the morphogenesis and/or the maintenance of epithelia.

I. EPITHELIAL ARCHITECTURE

Epithelia form the covering of all body surfaces, and they are the major component of glands. They often perform specialized functions, such as filtration (kidney tubules), absorption (intestine), secretion (exocrine glands), or the provision of an impermeable barrier (skin). Epithelial cells have a characteristic apical–basal polarity, with strong lateral cell–cell contacts and physical

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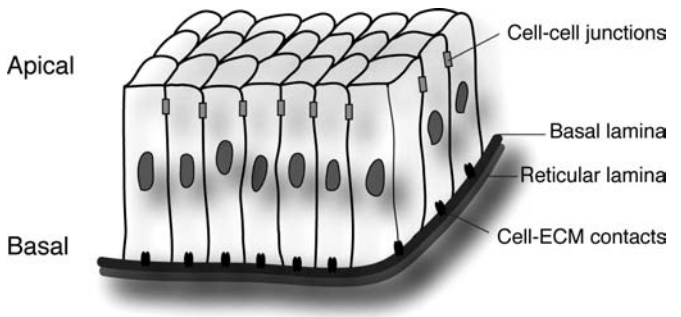

FIGURE 20.1 **Epithelial architecture.** A schematic representation of a typical columnar epithelial layer. The cells have a clear apicobasal polarity, and they are connected to each other via cell–cell junctions; the basal lamina is linked to the cells via cell–ECM contacts.

interaction with a basal lamina (Figure 20.1). Depending on their configuration and shape, epithelial cells are referred to as *squamous* (irregular and flattened shape), *cuboidal* (cube-like), or *columnar* (column shaped). Some epithelia are multilayered (e.g., vertebrate skin), whereas others have a single layer (e.g., *Drosophila* tracheae).

A. Cell-to-Cell Contacts

Neighboring cells in an epithelium are bound together by transmembrane molecular complexes (Figure 20.2), which direct communication between cells and maintain tissue integrity and function. In vertebrate epithelial cells, the junctional complexes consist of tight junctions (most apical), adherens junctions (apicolateral), and desmosomes (basolateral; see Figure 20.2). Although invertebrate epithelial cells are also connected by adherens junctions, which are comprised of evolutionarily conserved components, they lack tight junctions and desmosomes. Instead, invertebrate epithelial cells are connected apicolaterally by the marginal zone and basolaterally by septate junctions (see Figure 20.2). Each of these multiprotein complexes has both a structural function and a role in signal transduction. The junctions are dynamic and highly regulated both during development and in the adult, particularly during wound healing.

Adherens junctions are enriched in epithelia that undergo strong contractile forces during embryonic development or tissue repair. A key component is the transmembrane protein, E-cadherin, which exhibits homotypic, calcium-dependent binding to E-cadherin in the neighboring cell membrane. Intracellularly, E-cadherin is bound by β-catenin, which in turn binds to α-catenin. α-Catenin can also interact with F-actin and regulate the assembly of actin filaments. It has recently been shown that α-catenin functions as a molecular switch, which either binds E-cadherin/β-catenin (as an α-catenin monomer) or F-actin (as α-catenin dimers; Drees et al., 2005; Gates and Peifer, 2005; Yamada et al., 2005). α-Catenin dimers can directly regulate actin filament organization by suppressing Arp2/3-mediated actin polymerization. By contrast, α-catenin monomers prefer E-cadherin/β-catenin complexes, which suggests that adherens junctions are more dynamic than previously thought (Drees et al., 2005). It should be noted that the junctional complexes do not function in isolation; for example, α-catenin can bind to a component of the

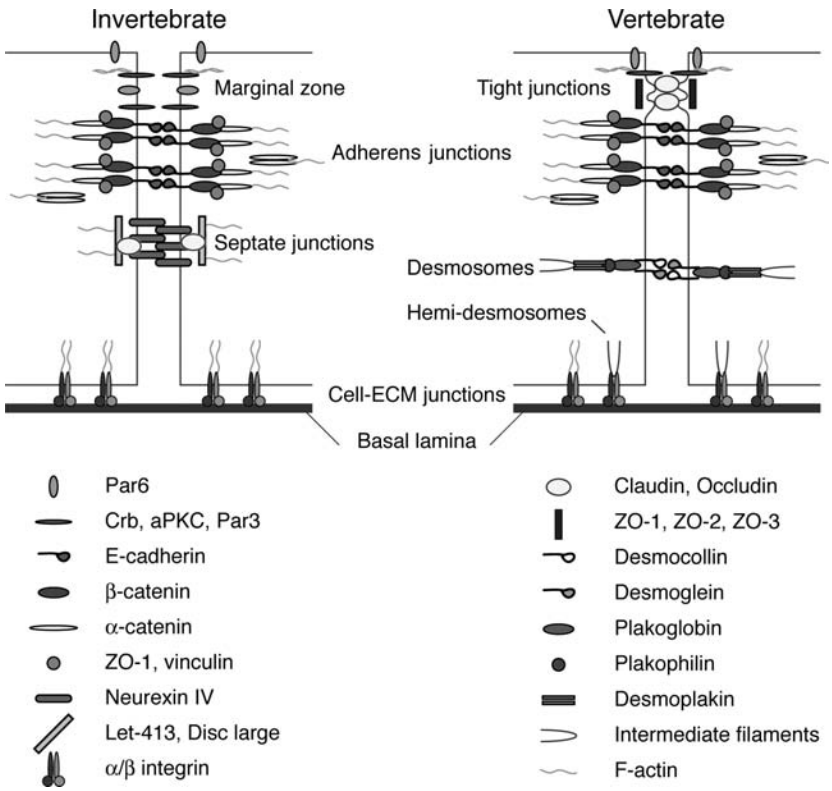

FIGURE 20.2 **A comparison of invertebrate and vertebrate cell–cell and cell–ECM junctions.** Both invertebrate and vertebrate epithelial cells are connected via E-cadherin/catenin-based adherens junctions, which are conserved both structurally and functionally. They also share integrin-based cell–ECM connections. Septate junctions are specific to invertebrates, but they share some gating functions with vertebrate tight junctions. Desmosomes and hemidesmosomes are linked to intermediate filaments, and they are only found in vertebrate cells. (See color insert.)

tight junctions (ZO-1), thereby regulating intracellular signal transduction (Vasioukhin and Fuchs, 2001).

Many studies have investigated the importance of adherens junctions in proper epithelial assembly and signal transduction (Jamora and Fuchs, 2002). β-Catenin, for example, has a dual role in cell adhesion and signal transduction via the Wingless/Wnt signaling pathway. β-catenin dynamically moves between two pools: the free, cytoplasmic pool and the pool that is bound to E-cadherin and α-catenin. After the phosphorylation of Tyr^{654} by c-Src, E-cadherin binding is lost. In the absence of Wnt signaling, free β-catenin is rapidly degraded via the adenomatous polyposis coli (APC) pathway. When Wnt signaling is active, β-catenin is phosphorylated on Tyr^{142}; it is translocated to the nucleus, and it acts as a transcriptional coactivator with T-cell factor/lymphoid enhancer factor (TCF/LEF) and BCL9–2 (Brembeck et al., 2006). Expression in the mammary gland of a dominant-negative version of β-catenin inhibits the formation of mammary epithelial tissue and induces apoptosis (Tepera et al., 2003).

Desmosomes function together with adherens junctions to establish and maintain vertebrate epithelial sheets (Cheng et al., 2005). The transmembrane

components of desmosomes are desmogleins and desmocollins, which are members of the E-cadherin superfamily of proteins. As for E-cadherin, these components assemble into heterodimeric complexes in a calcium-dependent manner and bind to homotypic complexes on neighboring cells. The desmosomal cadherins bind to plakoglobin (a close homologue of β-catenin) inside the cell. Plakoglobin, in turn, binds to desmoplakin (a spectrin family member) and plakophilin (a relative of ZO-1) to connect the complex to intermediate filaments. Thus, several of the core components of the desmosomal complex are evolutionarily related to those of the adherens junction. Furthermore, both complexes interact with the cytoskeleton: one, the adherens junction, is specialized to interact dynamically with the actin-based cytoskeleton, whereas the other, the desmosome, interacts with intermediate filaments.

Desmosomal defects have been implicated in tissue fragility syndromes in the skin and the heart. For example, a mutation that truncates the desmoplakin protein, thereby eliminating the C-terminal tail, results in the collapse of the intermediate filament network (Norgett et al., 2000). In the heart, this causes left ventricular cardiomyopathy (a heart muscle disease in the left ventricle); in the skin, the result is striate keratoderma (a skin disorder consisting of a growth that appears horny) of the palmoplanar epidermis.

Tight junctions, or zonula occludens, reside most apically in the lateral membranes that connect vertebrate epithelial cells (see Figure 20.2). As for the other cell–cell adhesion complexes, they include transmembrane proteins (claudin, occludin, and/or junctional adhesion molecules) that exhibit homotypic interactions with those on adjacent cells. The cytoplasmic tails of these proteins bind directly to cytoplasmic adaptor proteins, such as ZO-1, -2, or -3, Par3, and Par6 (the Pars are discussed in more detail later in this chapter). These adaptors in turn bind to F-actin. Tight junctions recruit signaling proteins (e.g., aPKC [which binds to occludin], Par6, Par3) and transcriptional regulators (e.g., AP-1 [which binds ZO-2]).

Major roles of tight junctions are to restrict diffusion within the plasma membrane, thus preventing the mixing of apical and basolateral lipids, and to prevent the movement of fluids across the apical–basal axis of epithelia (reviewed by Matter et al., 2005). Tight junctions also bind the Crumbs complex as well as the Par3/Par6/aPKC complex to regulate cell polarity (described later). RhoA, Rac1, and Cdc42 physically interact with tight junctions; tight-junction–associated proteins regulate the activation of these small GTPases, which in turn regulate the actin-based cytoskeleton.

Phenotypic analyses of tight junction components have produced only weak phenotypes, probably as a result of functional redundancy. For example, the removal of ZO-1 in mouse epithelial cell clones causes a delay in junction assembly, but it is not essential for junction formation (Umeda et al., 2004). Similarly, RNA-interference–mediated knockdown of ZO-1 in Madin–Darby canine kidney (MDCK) cells causes a delay of apical ring remodeling when the cells begin to establish contacts with one another (McNeil et al., 2006). It is worth noting here that epithelial tight junctions have structural and functional similarity to endothelial and neuromuscular junctions as well as to neural synapses and that their formation is regulated in a similar way.

Although structures that resemble desmosomes and tight junctions are absent from invertebrate epithelia, bilaterian invertebrate epithelial cells contain septate junctions basolaterally and a marginal zone apicolaterally (Fei et al., 2000; Knust and Bossinger, 2002). These are to some extent

functionally (but not spatially) homologous to tight junctions and desmosomes, respectively (see Figure 20.2). Indeed, the homology between the vertebrate and the invertebrate junctions extends in part to the molecular level, because vertebrate tight junctions share some components with the invertebrate marginal zone and septate junctions (e.g., Par3, Crumbs, and ZO-1). Because intermediate filaments are absent from invertebrates, the intracellular interactions of septate junctions (which are with F-actin) are distinct from those of desmosomes (which are with intermediate filaments).

The transmembrane component of septate junctions is neurexin IV, a protein with epidermal growth factor (EGF) repeats and laminin G domains. In Drosophila, the cytoplasmic tail of neurexin interacts with Scribble and Discs large, which are PDZ-domain–containing proteins (named for postsynaptic density, discs large and ZO-1 in which the domain was first described) that function to regulate intracellular signaling. These molecules (but not the actual junctions) have functional homologs in *C. elegans* and some vertebrates, where they are localized to the basolateral plasma membrane (Knust and Bossinger, 2002; Tepass et al., 2001).

B. Cell-to-Extracellular Matrix Contacts

The basal region of epithelial cells directly contacts a structure known as the *basement membrane,* which is a specialized form of ECM that envelops epithelia, muscle fibers, and nerves. The basement membrane is composed of two layers: the basal lamina, which is in direct contact with the cells and comprised of glycoproteins like collagen type IV and laminin, and the reticular lamina, which is composed largely of fibrillar collagens. The basal lamina is connected to epithelial cells via a family of transmembrane proteins called *integrins*. The cytoplasmic tails of integrins interact with F-actin, integrin-linked kinase, talin, and Src, thereby creating structural and signaling bridges between the ECM and the epithelium. Signals and, thus, regulatory information, can travel both ways: from the extracellular environment to the inside of the cell and vice versa.

Integrins are heterodimeric transmembrane proteins that are composed of α and β subunits and that are found in organisms from sponges to mammals. Each subunit spans the plasma membrane once, leaving a short cytoplasmic tail and a long extracellular polypeptide. Different α subunits can bind to different β subunits, thereby creating tissue-specific as well as structurally and functionally distinct integrins; these range from two in nematodes and primitive bilateria to 24 in mammals. The integrins can be subdivided according to their ligand-binding ability: one class recognizes the RGD tripeptide sequence found in proteins, such as tiggrin in Drosophila or fibronectin in vertebrates; another class binds to laminin receptors in the basement membrane, as is the case for some vertebrate integrins (Hynes, 2002).

Integrin-based cell–ECM contacts (focal complexes, focal adhesions, fibrillar adhesions, podosomes, and invadopodia) include distinct components, and they vary in size and organization, depending on their cellular and tissue context. Focal complexes are integrin-based, and they can mature and grow into focal adhesions on hard matrices through the recruitment of vinculin, talin, and paxillin. On soft matrices, focal complexes evolve into fibrillar adhesions that, unlike focal adhesions, contain high levels of tensin (Zamir et al., 1999). Thus, the rigidity of the matrix feeds back to control

the components of the adhesion complex, its properties, and its functions (Zamir and Geiger, 2001). Podosomes and invadopodia are structurally distinct from adhesions, and they are formed by a core of F-actin and actin-associated proteins, which are surrounded by a ring of integrin and integrin-coupled proteins, such as talin and vinculin. Both podosomes and invadopodia have been implicated in matrix degradation and cell adhesion dynamics (Buccione et al., 2004; Linder and Aepfelbacher, 2003; Linder and Kopp, 2005).

Depending on the extracellular ligand to which the integrin binds, as well as intracellular events (e.g., binding to talin), integrin may undergo a conformational change that triggers intracellular or extracellular signaling (Ginsberg et al., 2005; Hynes, 2002). Thus, integrins serve as a gate for cell–ECM communication, with associated changes in epithelial architecture and cellular behavior.

Because integrins comprise conserved components of most cell–ECM contacts and form the conduit for signaling between epithelial cells and the ECM, the elucidation of integrin function and regulation has been essential to understanding cell migration, tissue morphogenesis, tissue regeneration, cell proliferation, and cancer metastasis. Mutations or knockouts of different integrin subunits lead to a plethora of phenotypes in epithelial organization and in morphogenetic events that are driven by epithelial changes (Ginsberg et al., 2005; Hynes, 2002). These include reduced branching morphogenesis in lungs, skin blistering (when the mouse α3 subunit is affected), abnormal embryonic gastrulation (when the mouse β1 subunit is mutated), defects in embryonic gut morphogenesis (when both β integrins are mutated in Drosophila; Devenport and Brown, 2004), and defects in coordinated epithelial cell shape changes and migration over the yolk sac membrane during germ band retraction and dorsal closure (when β1 integrin levels are reduced in Drosophila; Narasimha and Brown, 2004; Reed et al., 2004).

C. Establishment and Maintenance of Epithelial Cell Polarity

The establishment and maintenance of the apical–basal polarity of epithelial cells require spatial regulation of the synthesis and deposition of components of the plasma membrane, cell–cell, and cell–ECM adhesion complexes, together with coordinated regulation of the organization of the F-actin–based cytoskeleton (reviewed by Le Bivic, 2005). These asymmetries are established via a combination of spatially restricted internal cellular components and external cues.

The first signs of epithelium formation and cell polarity in mammalian embryos are detectable at the eight-cell stage, when E-cadherin is synthesized and forms the first cell–cell junctions (De Vries et al., 2004). Epithelia do not develop in the presence of E-cadherin antibodies (Johnson et al., 1986), which suggests that E-cadherin is an essential—possibly the first—cue for cell polarization. However, recent studies of early Drosophila embryos, which have focused on the processes of cellularization and gastrulation, suggest that adherens junction formation, which is mediated by E-cadherin, is not necessary to establish polarization (Harris and Peifer, 2004). In that study, it was found that polarized epithelial cells form in mutants lacking E-cadherin *(shotgun)* or β-catenin *(armadillo)*.

Studies in Drosophila, *C. elegans,* and mammals have shown that several distinct protein complexes play important roles in the establishment and

maintenance of cell polarity. These include the aPKC/Par3/Par6 complex, the Crumbs/Stardust/Discs lost complex, and the Discs large/Scribble/Lethal giant larva (LGL) complex, all of which associate with the plasma membrane and exhibit a polarized distribution (Gibson and Perrimon, 2003; Tepass et al., 2001). Mutations in the genes that encode these proteins result in the loss of cell polarity in *Drosophila*, *C. elegans*, and mammals.

The Par proteins were first discovered in *C. elegans,* where Par3, Par6, and PKC-3 (the *C. elegans* aPKC homolog) were found to localize to the apical pole of epithelial cells in the epidermis and the digestive tract. Conserved orthologs were subsequently shown to have a similar distribution and function in Drosophila and vertebrates (reviewed by Nance, 2005). If Par3 is removed by RNA-mediated interference in the highly polarized epithelial cells of the worm's spermatheca, a subset of the cells loses polarity (Aono et al., 2004). LET-413 (*C. elegans*) and Scribble (Drosophila)—orthologous proteins that contain a PDZ domain and leucine-rich repeats—have been shown to be essential for apical localization of the Par complex. In *let-413* or *scribble* mutant epithelia, Par3, Par6, and PKC-3/aPKC are mislocalized laterally (Bilder and Perrimon, 2000; Legouis et al., 2000).

Research involving Drosophila and mammals has shown that the Crumbs/Stardust/Discs lost complex is localized apically, and that it regulates epithelial polarity by acting as an apical determinant. All three components contain PDZ domains, which interact with one another to assemble the complex. The complex in turn regulates the assembly and location of adherens junctions (Tepass, 1996; Tepass et al., 1990; Wodarz et al., 1995).

Another complex that is conserved in humans, Drosophila, and *C. elegans* comprises Discs large/Let-413, Scribble/LGL. Discs large is a member of the membrane associated guanylate kinase (MAGUK) family (ZO-1 is also a member), and it has three PDZ domains and one SH3 domain. Scribble, which is a PDZ-containing leucine-rich repeats and PDZ domains (LAP) family protein, colocalizes with Discs large and LGL in septate junctions. Mutations in any of the three components cause a lack of polarity in embryonic and wing disc epithelia. Discs large and Scribble act in the apical margin of the lateral membrane of epithelial cells to localize LGL. Because LGL is the Drosophila homolog of yeast t-SNARE, it has been hypothesized that LGL promotes the fusion of vesicles to the apical membrane in a Discs large/Scribble-dependent manner (Bilder et al., 2000; Humbert et al., 2003; Wodarz, 2002).

As mentioned previously, junctional complexes are linked to the cellular cytoskeleton. Thus, it is not surprising that the latter has been implicated in the establishment and maintenance of epithelial cell polarity. In particular, members of the Rho family of small GTPases (Cdc42, Rac and Rho, which are molecular switches that regulate the cytoskeleton) are required to maintain cell polarity and epithelial architecture. Cdc42 and Rho, for example, function to stabilize adherens junctions and to regulate the polarized transport of vesicles, whereas Rho also has a role in the Src/Fyn-dependent tyrosine phosphorylation of β-catenin (Calautti et al., 2002), the function of which was discussed above.

D. Evolution of Epithelia

Organized epithelia form the building blocks for complex tissue and organ development in eumetazoans. Epithelia are apparent early in the metazoan

stem, and they possess many conserved structural and molecular components all the way to mammals. Most strikingly, the zonula adherens complex and the ECM components are highly conserved (described previously; see Figure 20.2; van der Flier and Sonnenberg, 2001). The fundamental mechanisms and machinery used to establish apical–basal polarity of epithelial cells are also conserved in nematodes, Drosophila, and mammals (described previously; Suzuki et al., 2001; Wodarz et al., 2000).

In contrast with eumetazoa, the sponges possess more primitive epithelia that lack belt-forming junctions and thus are much more permeable than those of eumetazoa (Tyler, 2003). The epithelial cells in sponges also display polarization, which implies that cell polarization formed the first step in (and may have been a prerequisite for) the evolution of animal epithelia. The next steps may well have involved the following: (1) the appearance of integrins and thus connectivity to the ECM, which enabled the formation of complex and stable multicellular structures; and (2) the origin of cadherin-like molecules, which would have permitted the formation of a stable, sheet-like arrays of cells (Tepass et al., 2000). Of note in this regard is the fact that adhesion proteins related to cadherins (e.g., C-type lectin) are found in sponges and in eukaryotes outside of the metazoa (Harwood and Coates, 2004).

Later steps would have led to the formation of adherens junctions through the recruitment of additional proteins by cadherin (e.g., catenins), thus enabling the formation of apicolaterally localized belt-like zonula adherens. Placozoa (represented by a single genus, *Trichoplax,* which has a contentious position in the evolutionary tree) have a single belt, whereas some nematode worms, including *C. elegans*, have multiple belts. The ability to seal different compartments within tissues from each other with septate-like junctions may have evolved next, because functional septate junctions are first detected in Cnidaria. Punctate contacts that resemble vertebrate tight junctions are found in Ctenophora, thereby placing their evolution as occurring simultaneous with or subsequent to that of septate junctions (reviewed by Tyler, 2003).

II. MODES OF EPITHELIAL MORPHOGENESIS

During development, simple epithelial sheets and mesenchymal cell masses are assembled into tissues and organs. Distinct modes of morphogenesis are used by different tissues and at different times during development. Epithelial cells undergo a variety of changes that include coordinated proliferation, shape change, local rearrangement, delamination, migration, and programmed death. In this section, we review some of the methods used to study epithelial morphogenesis, and we then focus on several different mechanisms by which simple epithelial sheets are assembled into complex and dynamic three-dimensional structures.

A. Methods of Analysis

Advances in imaging technology underlie most of the new methods for analysis of epithelial behavior *in vivo* or in tissue culture. Key microscopic methods include confocal fluorescence microscopy, two (or multi) photon microscopy, spinning disc microscopy, and single-plane illumination microscopy, each of

which is aimed at improving resolution and sensitivity and/or reducing the exposure of living cells to harmful radiation (reviewed by Keller et al., 2006). These microscopic methods have been complemented by the ability to tag endogenous proteins (or other macromolecules) *in vivo* with fluorescent tags (e.g., using red or green fluorescent protein [RFP or GFP] tags), thus enabling the real-time imaging of cells and macromolecular complexes inside of cells in living whole embryos, explants, or tissue culture.

Most recently, whole-genome analyses have revolutionized all aspects of biology by enabling the use of in silico techniques to find families of homologous genes as well as transcriptomic and proteomic methods that enable the identification of coregulated genes and proteins. The latter in particular promise to define new components of macromolecular complexes that function together in cell–cell and cell–ECM adhesion. Furthermore, studies of coordinate changes in gene expression in normal versus diseased tissues (e.g., in normal epithelia versus epithelial-derived cancers) have facilitated the identification of new molecular players and provided useful biologic markers for detection and prognosis.

Cultures of growing cells *in vitro* have contributed a great deal to our understanding of basic cellular processes. However, because epithelial morphogenesis occurs in the context of a three-dimensional living organism, the two-dimensional nature of traditional tissue culture has limited the usefulness of previous analyses. During the last decade, the development of methods to grow cells in three-dimensional arrays has revolutionized *in vitro* analyses of epithelial cell behavior (reviewed by Keller et al., 2006). Cells grown in three-dimensional matrices exhibit quite distinct adhesion properties as compared with the same cells grown in classic two-dimensional culture (Cukierman et al., 2001). To mimic *in vivo* events, cells grown in gel matrices can be exposed to growth factors, which induce morphogenesis. For example, MDCK cells exposed to hepatocyte growth factor form tubules after a few days (reviewed by Keller et al., 2006; Lubarsky and Krasnow, 2003).

Most important, however, have been *in vivo* analyses of epithelia in developing embryos. Initially, these were conducted on nongenetic models, such as the frog and chick. More recently, analyses have focused on genetic models such as *C. elegans, D. melanogaster, D. rerio,* and *M. musculus*. The nonmammalian models have a distinct advantage in this regard, because embryonic development occurs outside of the mother, thereby facilitating the live imaging of morphogenetic processes.

In the remainder of this section, we focus on several specific types of epithelial morphogenesis, and we highlight how *in vivo* imaging has combined with genetic analyses to yield deep insights into the mechanistic basis for these processes. The processes are discussed as if they are distinct, but it needs to be remembered that they very often occur simultaneously in the same tissue.

B. Local Cell Rearrangement: Convergence and Extension

During morphogenesis, coordinated local cell rearrangement is often used to change the shape of epithelia. Specifically, regulated cell intercalation can direct such alterations through a process known as *convergence* (the tissue narrows in one dimension) and *extension* (the tissue lengthens in an orthogonal dimension).

One of the best-known examples of convergence and extension is during germ-band extension of the Drosophila embryo. During this process, which lasts 2 hours, the epithelium comprising the germ band doubles in length (along the anterior–posterior axis) and halves in width (across the dorsal–ventral axis of the embryo). This is accomplished via the intercalation of cells derived from the dorsal–ventral axis of the epithelium between neighboring cells that lie along the anterior–posterior axis (Irvine and Wieschaus, 1994). During this process, cell–cell junctions along the dorsal–ventral axis disassemble, whereas new junctions are formed between the intercalated cells and their neighbors along the anterior–posterior axis.

The Drosophila embryonic ectodermal epithelium that undergoes convergence and extension is, in fact, polarized in the plane of the epithelium. For example, GFP-tagged Slam and its binding partner, myosin II, are localized at dorsal–ventral adherens junctions in the extending germ band (Bertet et al., 2004; Lecuit et al., 2002; Zallen and Wieschaus, 2004). Mutations in myosin II (an actin-based motor) or the inhibition of Rho kinase (Rho kinase normally induces myosin activation) block germ-band extension (Bertet et al., 2004). In contrast with Slam and myosin II, Par3/Bazooka is concentrated at anterior–posterior junctions, which lack myosin II. These observations led to the model that say that Rho kinase and myosin II promote dorsal–ventral junction disassembly, whereas Par3/Bazooka stabilizes the anterior–posterior junctions, thereby permitting cell intercalation (reviewed by Baum, 2004).

Interestingly, during elongation of the *C. elegans* embryo, as in Drosophila, Rho kinase activates myosin II in dorsal–ventrally oriented junctions. However, in *C. elegans,* the junctions remain unbroken, and intercalation is inhibited; this results in embryo elongation via cell shape changes rather than convergence and extension (Wissmann et al., 1997). Thus, the same molecules regulate epithelial morphogenesis, but they do so via distinct cellular mechanisms.

Convergence and extension are used time and again during vertebrate embryogenesis. For example, during Xenopus gastrulation, cells in the mesoderm intercalate between their neighbors following lamelliform protrusion formation, whereas during zebrafish gastrulation cells undergo mediolateral elongation and intercalation (reviewed by Solnica-Krezel, 2005). A noncanonical Wnt pathway, which was first defined in studies of planar cell polarity in Drosophila, is essential for mediolateral cell polarization, which underlies vertebrate convergence and extension. In this pathway, the transmembrane protein Frizzled signals through Dishevelled, Rho-family GTPases, and Jun kinase (JNK) to stabilize cell protrusions and to regulate the orientation of the cells (reviewed by Solnica-Krezel, 2005). In zebrafish, *wnt11* and *wnt5* mutations result in shorter and broader embryos, because convergence and extension fail (Heisenberg et al., 2000). On the basis of gain- and loss-of-function phenotypes, many other molecules have been implicated in vertebrate convergence and extension; these include E-cadherin (in zebrafish), transforming growth factor (TGF)–βs (in zebrafish and Xenopus), and Snail (in mouse). The detailed mechanisms through which these molecules participate in convergence and extension remain unclear.

C. Cell-Shape-Change–Driven Epithelial Sheet Morphogenesis: Epiboly

Although local cell rearrangement drives convergence and extension, there are also many examples during both vertebrate and invertebrate development

during which epithelial sheet morphogenesis is accomplished largely or exclusively by cell shape change without significant changes in cell position relative to their neighbors. Best understood are the dorsal closure of the Drosophila embryo and the ventral enclosure of the *C. elegans* embryo, both of which are examples of the spreading of a bounded epithelium over a substrate, which is a process that is also known as *epiboly* (Chin-Sang and Chisholm, 2000; Martin and Parkhurst, 2004). During dorsal closure and ventral enclosure, cells in epithelial sheets on the left and right side of the embryo change shape, thereby leading to closure and fusion of the epidermis around the internal tissues of the embryo. It has been found that similar cellular and molecular events underlie mammalian wound healing; thus, these invertebrate processes serve as models for understanding the wound-healing process.

Drosophila dorsal closure is accomplished via coordinated cell shape changes within the epidermal epithelium on the left and right sides of the embryo. Sophisticated techniques applied to live Drosophila embryos have included the laser ablation of cells (Kiehart et al., 2000) and/or *in vivo* confocal microscopy to follow GFP-labeled molecules that mark cell membranes and the cytoskeleton (Reed et al., 2004). These methods have been combined with genetic analyses to dissect the cellular and molecular events during dorsal closure (Harden, 2002; Jacinto et al., 2002). It has been found that an F-actin contractile "cable" forms in the apicodorsal region of the "leading edge" epidermal cells, which are the dorsal-most cells within this epithelium. Communication between the leading edge cells and the more lateral epidermal cells results in the coordinated elongation of cells along the dorsal–ventral axis, with shortening across the anterior–posterior axis. There are also strong interactions between the leading edge cells and the underlying amnioserosa, which is an epithelium that undergoes complementary shape changes (i.e., contraction across the dorsal–ventral axis and elongation along the anterior–posterior axis; Figure 20.3, A and B). Together, these processes bring the left and right epidermal sheets together at the dorsal midline of the embryo, where signaling and contact between filopodia and lamellipodia from the left and right sides ensure in-register fusion of the sheets. These subcellular processes subsequently regress, and permanent adherens junctions form.

Epithelial closure requires orchestrated F-actin assembly and function. Thus, mutations in the small GTPases (Rho, Rac, and Cdc42) result in dorsal closure defects in Drosophila (Hall and Nobes, 2000). For example, when Rac function is eliminated, F-actin fails to accumulate at the leading edge, and both filopodia and lamellipodia are severely reduced (Hakeda-Suzuki et al., 2002). These small GTPases are also upstream activators of the Jun and p38 mitogen activated protein (MAP) kinase pathways. Regulation of JNK signaling in the epidermal leading edge and the underlying amnioserosa has also been shown to be essential for Drosophila dorsal closure: mutations in components of this pathway result in "dorsal open" embryos. In the amnioserosa, JNK signaling must be downregulated to enable closure to occur (Reed et al., 2001). By contrast, an active JNK cascade is essential in the epidermal leading edge. AP-1 (Jun/Fos) activation in these cells induces transcription of at least two downstream genes: *dpp* (TGF-β) and *puckered*, which encodes a JNK phosphatase that is a negative regulator of the JNK pathway (Martin-Blanco et al., 1998). Filopodium formation by the leading edge cells depends on both the JNK pathway and the Rho-family GTPases.

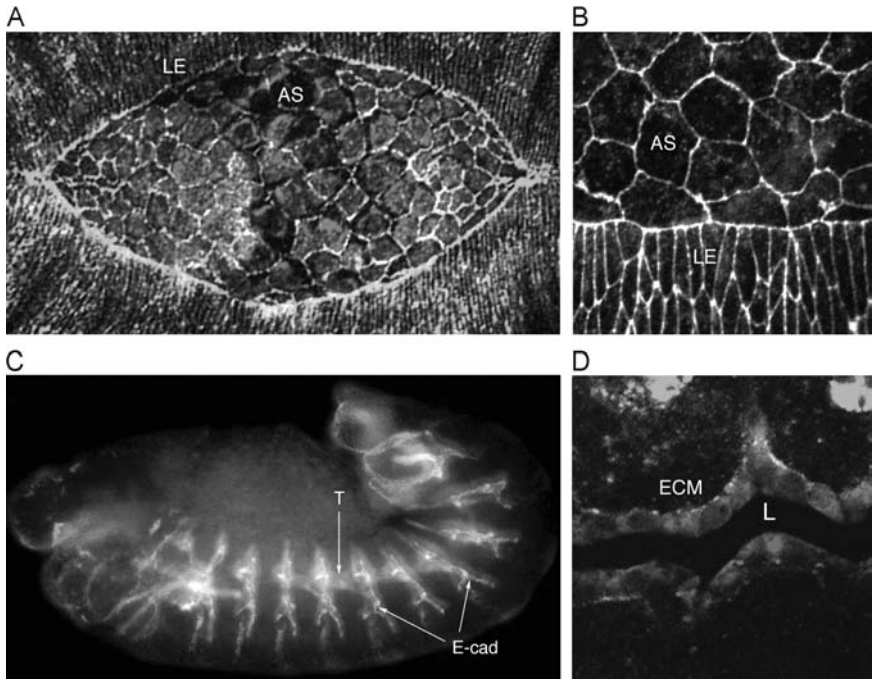

FIGURE 20.3 **Epithelial morphogenesis in Drosophila embryos.** Drosophila embryos are shown during dorsal closure (**A** and **B**) and during the branching morphogenesis of tracheal tubes (**C** and **D**). **A,** Dorsal view of a mid stage embryo (stage 14) showing dorsal–ventrally elongated epidermal epithelial cells, which are closing over the squamous amnioserosal epithelium. Cell membranes are highlighted with antiphosphotyrosine antibody *(green)*, which highlights the leading edge of the epidermis adjacent to the amnioserosa. The amnioserosa is marked by high levels of D-Fos *(magenta)*, which is cytoplasmic at this stage, because JNK signaling has been downregulated (Reed et al., 2001). **B,** High-magnification view of the boundary between the squamous amnioserosal epithelium *(AS)* and the elongated, closely packed cells of the leading edge *(LE)* epithelium of the dorsal epidermis. Cell membranes are highlighted with antiphosphotyrosine antibody. **C,** Lateral view of a mid stage embryo (stage 13) showing tracheal tubes (*trachealess* enhancer trap *1-eve-1 [red, T]*). E-cadherin is concentrated at the apical poles of tracheal epithelial cells (visualized with anti–E-cadherin antibody *[green, E-cad]*). **D,** High-magnification view of part of the dorsal tracheal trunk, showing the tracheal epithelial cells *(red*; *trachealess* enhancer trap *1-eve-1)*, the basal ECM in *(green;* anticollagen IV antibody *[ECM])*, and the lumen of the tube *(black, L)*. (Panels **A** and **B** courtesy of Dr. Bruce Reed. See Wilk et al. [2000; 2004] and Reed et al. [2001] for details. See color insert.)

Genome-wide approaches have been used to identify additional genes that are regulated by the JNK pathway in Drosophila. Serial analysis of gene expression (SAGE) studies has identified numerous candidates, including cell adhesion molecules and cytoskeletal regulators such as profilin (Jasper et al., 2001; see http://www.sagenet.org for more information). Subsequent analyses of *chickadee* mutant embryos (which lack profilin function) showed that they fail to undergo the cytoskeletal rearrangements required for dorsal closure. The further application of genomic methods, such as SAGE and tissue-specific gene expression profiling, will be a fruitful approach to the identification of new players in biologic processes such as dorsal closure.

Analogous to dorsal closure in Drosophila is ventral enclosure in *C. elegans,* which is a dynamic process by which the outer epithelia of the developing worm enclose the underlying tissues. This process takes place in two

distinguishable steps. First, two pairs of cells in the epithelium migrate toward and meet at the ventral midline; filopodium formation is clear during this stage. Second, eight pairs of posterior contralateral cells move together and form junctions. Actin force generation is essential for this step; mutations in any of the components of the Arp2/3 complex, which is essential for the nucleation of new actin filaments, result in a lack of ventral enclosure. In mutant embryos, filopodium formation is affected, and cell migration is inhibited (Sawa et al., 2003). The underlying neuroblasts are required as a substrate for ventral enclosure, during which ephrin-mediated signaling plays a key role (Chin-Sang and Chisholm, 2000), just as the underlying amnioserosa is required for dorsal closure in Drosophila (Reed et al., 2001; Reed et al., 2004).

Interestingly, similar regulation to that seen in flies and worms is likely to control vertebrate epithelial sheet morphogenesis. For example, a tissue-specific Jun knockout in the mouse eyelid epithelium results in defects in eyelid morphogenesis (Li et al., 2003; Zenz et al., 2003), whereas mutations in mouse JNK1 and JNK2 genes lead to defects in neural tube closure (described later; Sabapathy et al., 1999). Some types of mammalian wound healing also exhibit parallels with embryonic epithelial closure in flies and worms; these include embryonic wound healing as well as adult cornea and gut repair (Jacinto et al., 2001). During these processes, there is collaboration between actin cable "purse-string" forces and lamellipodia-driven cell migration to close the wound. Molecularly, mammalian tissue repair and Drosophila dorsal closure share many components, including Rho-family GTPases and JNK (Jacinto et al., 2001).

D. Tube Formation

Tubes, which are used to transport vital fluids and gases, are found in all major vertebrate organs, including the kidneys, lungs, and the vascular system, as well as in the tracheal system of arthropods. They are almost always composed of an epithelium with cells that are oriented with their apical poles facing the inside of the tube and their basal poles facing the outside. Tubes vary in size and design across different organisms, ranging from less than 0.1 μm in insect tracheae to more than 20 cm in an elephant's gut. They also range in cross-sectional composition from a single cell to several thousand cells. Despite these differences, there appear to be shared mechanisms underlying tube morphogenesis, including a key role for apical membrane biogenesis in the expansion of tube diameter (Lubarsky and Krasnow, 2003; see Chapter 21).

Tube morphogenesis can be classified into five main classes (reviewed by Lubarsky and Krasnow, 2003):

1. *Wrapping:* The tube develops from an epithelial sheet that curls up until its edges meet and then seal. Examples are found during the formation of the neural tube of many vertebrates.
2. *Cavitation:* The tube originates from a solid cylinder in which the central or core cells are eliminated. An example is salivary gland morphogenesis in the mouse.
3. *Cord hollowing:* A solid cylinder opens up to form a tube without any cell loss. Examples are the *C. elegans* gut and the Drosophila heart.
4. *Cell hollowing:* A single cell changes shape to form a lumen within or through the cell. Examples include capillary endothelial cells in mammals

and tertiary tracheal cells in Drosophila, which form a lumen in their cytoplasm that is open at both ends.

5. *Budding:* From an epithelial sheet, cells invaginate orthogonal to the plane of the epithelium, thereby forming a tube as the bud grows. Examples are the branches in mammalian lungs and Drosophila tracheae.

It is thought that cavitation, cord hollowing, and cell hollowing occur via a common cellular mechanism. The current model suggests that, after the polarization of the cells in the developing tube, the cells generate vesicles that carry apical membrane antigens. These vesicles fuse with the apical membrane to form a lumen that expands by further secretion. During cavitation, the interior cells themselves do not become polarized, and they are eliminated by cell death within the developing lumen. However, it should be noted that, in a three-dimensional *in vitro* model, the inhibition of cell death does not prevent lumen formation (Debnath et al., 2002).

Neural tube formation or neurulation has been studied for decades in vertebrates, including, most recently, the zebrafish *D. rerio*. High-resolution microscopy, advanced genetics, and a transparent embryo that develops rapidly and external to the mother have made this a particularly good model system. To form the neural tube, the neural plate (a specialized dorsal ectoderm) develops bilateral folds that elevate and fuse at the dorsal midline; this process is known as *neural tube closure*. The fusion of the midline is characterized by lamellipodial cell protrusions that interdigitate at the site of adhesion of the left and right neural folds.

The noncanonical Wnt pathway is required for the initiation of neural tube closure by promoting the convergence of neural progenitors to the dorsal midline. Components of this pathway include Van Gogh-like2 (Strabismus in Drosophila), which interacts with Dishevelled (an adherens junction component) and Flamingo/Celsr-1 (which binds to Frizzled in the plasma membrane). Live imaging has revealed that, as the neural tube develops, more apically located cells lose most—but not all—of their contact with the basal lamina and that they round up and divide (Ciruna et al., 2006). The daughter cell is incorporated into the epithelium on the opposite side of the neural tube, and it subsequently establishes apical–basal polarity. The mother cell reintegrates into the epithelium on the ipsilateral side of the tube. Loss of Van Gogh-like2 abolishes polarization of the daughter cells and their integration into the neuroepithelium, thereby leading to a mass of unpolarized cells filling what would have been the lumen of the tube. Thus, planar cell polarity signaling couples cell division and epithelial morphogenesis during neural tube formation.

E. Branching Morphogenesis

Although simple tubes are unbranched (e.g., the neural tube), in many organ systems, tubes undergo complex and stereotypical branching patterns (e.g., mammalian lungs and kidneys; the Drosophila tracheal system; see also Chapter 21). As a result of sophisticated genetic tricks, large-scale genetic screens, the ease of gene cloning and molecular analysis, and the ability to conduct high-resolution microscopy of living embryos, most of our basic knowledge of branching morphogenesis has come from studies of the tracheal system of Drosophila (Ghabrial et al., 2003; Lubarsky and Krasnow, 2003).

The specification of tracheal cell fate is initiated by the basic helix-loop-helix (HLH) transcription factor, Trachealess (Trh), which is expressed in clusters of ectodermal epithelial cells on the left and right sides of the developing embryo (Isaac and Andrew, 1996; Wilk et al., 1996). These clusters of Trh-positive cells subsequently invaginate, migrate, and fuse in an E-cadherin–, β-catenin–, Rho-A–, and F-actin–dependent way to create a complex interconnected branched tubular network (Figure 20.3, C and D). After primary branch formation, each segmental tracheal unit fuses with adjacent units to form a continuous open tube. This process is controlled by the fusion cells, which reside at the branch tips. E-Cadherin and β-catenin form an F-actin–rich bridge between the tip cells, which facilitates fusion (Lubarsky and Krasnow, 2003; Uemura et al., 1996). Rho-A acts as a negative regulator of the fusion process.

Branching morphogenesis in the tracheal system is controlled by the fibroblast growth factor (FGF) signaling pathway: the FGF ligand, Branchless (Bnl), is expressed in spatially restricted clusters of ectodermal and mesodermal cells near the placode (Metzger and Krasnow, 1999). Binding to the FGF receptor, Breathless (Btl), which is expressed in the tracheal epithelium, induces branch formation and migration toward the ligand-expressing cells. *In vivo* confocal microscopy has shown how Bnl induces filopodia formation at the tips of migrating tracheal cells (Ribeiro et al., 2002; Sato and Kornberg, 2002).

In addition to the spatial information provided by the FGF pathway, Decapentaplegic (Dpp/TGF-β), the canonical Wnt pathway and the EGF pathway control the migration of specific branches (Llimargas and Casanova, 1999; Wappner et al., 1997). For example, Dpp signaling directs tracheal cell migration along the dorsal–ventral axis, whereas anterior–posterior migration is controlled by Wnt. FGF is required for filopodium formation, whereas Dpp is important for branch outgrowth. The FGF pathway acts together with cell-surface proteins that control cell–cell and cell–ECM interactions. For example, the expression of αPS1 integrin in the middle part of the developing placodes and of αPS2 integrin in the visceral mesoderm promotes visceral branch migration (Boube et al., 2001).

During tracheal morphogenesis, a dramatic expansion of the apical membrane of tracheal cells leads to an increase in tube diameter in the dorsal trunk (described previously). Genetic screens have shown that many genes that encode components of the septate junctions (e.g., *sinous, mega*) are also important for tube expansion, possibly by regulating cell shape (Behr et al., 2003; Wu et al., 2004). In addition to the expansion of the lumen of larger tracheal tubes, which are formed by multiple cells, the secondary and tertiary branches of the tracheal system are formed by individual cells, which form tubes by the branching and shape change of individual cells. Molecules such as Dumpy and Piopio, which contain zona pellucida domains, form an apical extracellular matrix on the inside of the tracheal tubes, thereby converting intercellular adherens junctions into autocellular ones (Jazwinska et al., 2003).

The integrity of the tracheal epithelium as well as that of the amnioserosal and retinal epithelia in Drosophila is regulated in part by the zinc-finger transcription factor, Hindsight (Pickup et al., 2002; Reed et al., 2001; Wilk et al., 2000). The mechanism by which Hindsight regulates epithelial integrity is unknown; however, it has been shown that mutations in this gene exhibit

strong genetic interactions with those in several intercellular signaling pathways, including Notch/Delta, JNK, and Dpp/TGF-β, as well as with components of the extracellular matrix, such as collagen IV and laminin (Wilk et al., 2004). Thus, it is possible that Hindsight regulates tissue-specific expression or function of one or more of these key components of epithelial structure and function.

F. Epithelial-to-Mesenchymal Transitions in Development

An epithelial-to-mesenchymal transition (EMT) is a stepwise process that transforms polarized, attached, stationary epithelial cells into unpolarized, detached, motile cells (i.e., mesenchyme; Figure 20.4). EMTs occur during normal embryonic development during the course of the morphogenesis of various organs, including the heart and the peripheral nervous system (Huber et al., 2005; Thiery, 2002). During an EMT, epithelial cells undergo cytoskeletal reorganization, organelle redistribution, and changes (often, loss) of cell–cell junctions. Reciprocally, the opposite changes, when imposed on mesenchymal cells, can cause them to gain (or regain) epithelial fate via a mesenchymal-to-epithelial transition.

Mesoderm development is an excellent system in which to study the molecular regulation of an EMT. In Drosophila embryos, for example, after invagination into the ventral furrow, mesodermal cells dissociate from the epithelium and migrate dorsally to form a monolayer underlying the ectoderm. As they migrate, the mesodermal cells change their morphology and extend protrusions. The Rho GTP exchange factor, Pebble, is essential for mesodermal cell migration as well as for their ability to form protrusions, which link the regulation of actin dynamics to cell migration during the EMT (Smallhorn et al., 2004). The EMT requires the collaboration of the DPP/TGF-β and the Ras/Raf/MAP kinase signaling pathways (see Figure 20.4). TGF-β signaling induces the expression of the zinc-finger transcription factor Snail, which

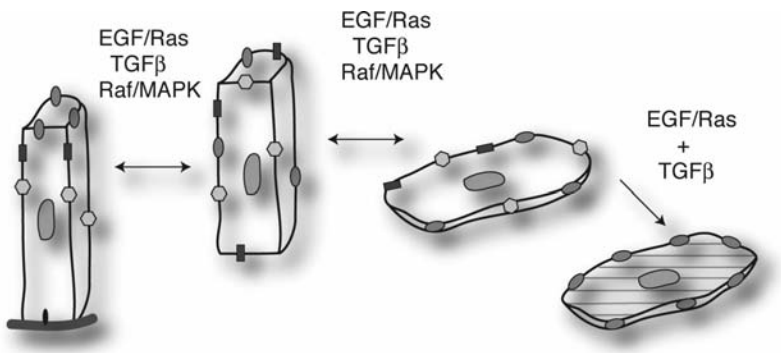

FIGURE 20.4 **EMT.** The cell on the left of the figure represents an epithelial cell that is attached to the basal lamina *(thick gray line)*, with cell-to-ECM connections *(black oval)*. It contains apical markers, E-cadherin, and basolateral markers. The transition into a mesenchymal cell, such as the one shown on the right of the figure, requires a series of steps that are controlled by different signal transduction pathways (shown above the arrows). During this process, cells lose polarity, change shape, and migrate through the ECM. Each step can be diagnosed using a combination of cell shape and molecular markers. At the end of the EMT, the cell acquires mesenchymal markers, such as vimentin, and it completely loses polarity.

represses the transcription of E-cadherin. The decrease in E-cadherin levels causes the disassembly of adherens junctions and the loss of cell–cell contacts (Oda et al., 1998).

Snail also represses E-cadherin expression during mouse gastrulation. In *snail* mutant mice, gastrulation is abnormal as a result of a defective EMT, which is caused by sustained E-cadherin expression (Nieto, 2002). During the EMT, E-cadherin levels at the adherens junctions are reduced not only at the level of transcription but also by increased internalization from the cell membrane, which occurs in response to elevated Src kinase activity (Avizienyte and Frame, 2005).

It should be noted that cell–ECM contact plays a key role in the maintenance of epithelial integrity. The loss of such contacts can induce a loss of integrity, cell death, and/or an EMT.

III. HUMAN EPITHELIAL DISEASES

Any failure in human epithelial morphogenesis or architecture results in developmental defects or diseases. These range from kidney and lung diseases to skin and tissue repair defects to cancers. The following sections present a few of the many examples of epithelial-related human diseases.

A. Defects in Epithelial Architecture or Integrity

The skin is a tough, protective, stratified epithelium that undergoes constant renewal and repair. Different keratins assemble into filaments to maintain the integrity of the skin epithelium. Cell adhesion junctions (desmosomes and hemidesmosomes) are the link between keratin filaments and the cell surface. Research in humans and animal models has shown that mutations in different keratin genes cause a phenotype that reflects the cell layer in which that keratin is expressed (Presland and Jurevic, 2002). For example, mutations in keratin-5 and keratin-14, which are normally expressed in basal epithelial cells, cause the delamination of the epithelial strata and a blistering phenotype known as *epidermolysis bullosa*.

When desmosomes are defective in the skin and oral mucosa as a result of either autoimmune or inherited diseases, they cause pemphigus diseases in which there is reduced cell–cell adhesion and a loss of keratin filaments, which causes blistering. The circulation of antidesmoglein antibodies is a hallmark of the disease; thus, it serves as a diagnostic test. *Pemphigus vulgaris* is an autoimmune disorder in which antibodies are produced against desmoglein 3 and which may cause desmosome disruption. This leads to the formation of intraepidermal clefts or bullae (McGrath, 2005).

Another example of skin epithelial malfunction is seen in patients with Kindler syndrome, which is caused by the loss of the epidermal protein, kindlin-1, which links the actin cytoskeleton to the extracellular matrix (White and McLean, 2005). In Kindler syndrome, there is neonatal blistering that progresses to poikiloderma (mottled pigmentation of the neck and chest) and sometimes to premature skin aging and photosensitivity.

Architectural defects also underlie abnormalities in the epithelia of internal organs. For example, if epithelial adherens-like junctions (which are

known as *glomerular slit diaphragms*) malfunction in the kidney, the result is proteinuria (an excess of protein in the urine) as a result of leakage across the renal glomerular epithelium (Reiser et al., 2000).

B. Defects in Epithelial Morphogenesis

Neural tube defects in humans occur at a frequency of 1 in every 1000 pregnancies worldwide. If closure fails, it can lead to anencephaly (the interior of the brain is exposed to the outside), craniorachischisis (the entire spine and most of the brain stay open), or spina bifida. A negative regulator of the Rho GTPase, p190RhoGAP, was the first gene directly implicated in human anencephaly. Studies in the mouse have shown that mutations in this gene lead to reduced apical constriction at the midline of the neural plate and ectopic actin accumulation, ultimately resulting in the mouse equivalent of anencephaly, which is known as *exencephaly* (Brouns et al., 2000).

Certain mammalian neural tube defects result from the failure of convergence and extension. For example, the disruption of planar polarity genes such as Dishevelled or Strabismus/Van Gogh1 in mouse embryos result in severe neural tube defects, which are similar to those reported in zebrafish (Ciruna et al., 2006).

One of the most common human morphogenetic disorders is autosomal dominant polycystic kidney disease (ADPKD). Mutations in PKD1 and PKD2 cause ADPKD, which involves cysts in the proximal and distal tubules as well as in the collecting ducts. Genetically, ADPKD mutations are recessive loss-of-function alleles. In heterozygotes, the somatic mutation of the remaining wild-type allele (i.e., the loss of heterozygosity) causes a chimeric mutant kidney with disrupted architecture and function of the renal tubules. Molecularly, PKD1 and PKD2 are large interacting transmembrane proteins that are expressed in the primary cilium at the apical surface of renal epithelial cells, where the proteins interact to form Ca^{++} channels. Normally, Ca^{++} influx through the channels trigger events that prevent tube expansion. Loss of PKD function leads to the failure of Ca^{++} ion influx into the epithelium and thus abnormal expansion of tube size (reviewed by Lubarsky and Krasnow, 2003). PKD functions are not limited to the kidney; in PKD mutants, liver and pancreatic cysts form, and blood vessels suffer aneurisms.

C. Defects in Epidermal-to-Mesenchymal Transitions

The link between EMT and tumorigenesis has been studied in detail in tissue culture and mouse models. As described previously, during an EMT, cells undergo a range of changes, including the loss of cell–cell junctions, the reorganization of the actin cytoskeleton, and the loss of polarity and migration through the basement membrane and tissues (see Figure 20.4). Metastasis and cell invasion correlate with what is referred to as a "complete EMT," during which all of the preceding steps occur, including the loss of epithelial markers (e.g., E-cadherin) and the gain of mesenchymal markers (e.g., vimentin).

During metastasis, cells from the primary tumor invade the tissue layers, migrate through the basement membrane, and disseminate and survive in the blood stream, later reestablishing a solid tumor in a distant host tissue. An EMT is required for the primary tumor cells to migrate through the basal

lamina into adjacent tissue layers. Actin regulatory components (e.g., cofilin, talin), adhesion molecules (e.g., integrins, paxillin), and matrix metalloproteases (e.g., MMP2) lead to the formation of invadopodia, which is the cellular structure in a primary tumor cell that invades the ECM.

Because of its key role in cell–cell adhesion, E-cadherin expression is an excellent diagnostic tool for tumors. In most types of breast cancer, if the cells retain polarized E-cadherin expression, the tumor can be classified as noninvasive, whereas a loss of polarized E-cadherin correlates with invasiveness (Berx and Van Roy, 2001). However, it should be noted that the correlation is less than perfect; regaining the expression of E-cadherin can also favor survival and metastasis (Cowin et al., 2005).

Studies of cell migration during normal embryonic development have been instrumental in our understanding of how cancer cells migrate (reviewed by Yamaguchi et al., 2005; see Chapter 19). Most recently, the live imaging of mice carrying GFP-labeled tumors (Condeelis and Segall, 2003) has enabled metastatic cells to be followed during their migration through the extracellular matrix to the blood vessels. During invasion, the cells follow cues provided by the EGF and EGF-like molecules, the latter of which are formed during the proteolysis of the ECM. In primary tumors, tumor-associated macrophages, which secrete EGF and which also remodel the ECM, have been linked to invasion and metastasis (Pollard, 2004).

SUMMARY

- Well-defined cell polarity, specific cell–cell junctions, and a clear connection to the basal lamina characterize epithelia.
- Many molecular components and regulators of epithelia are highly evolutionarily conserved in both structure and function, including junctional complexes (e.g., E-cadherins, catenins), cytoskeletal regulators (e.g., Rho-family GTPases), cell–ECM contacts (e.g., integrins, laminins, collagens), and signaling pathways (e.g., JNK, TGF-β, Wnt).
- Epithelial morphogenesis is accomplished by a combination of coordinated cell shape changes, rearrangements, migration, fate changes, and death.
- The live imaging of cells using GFP-tagged molecules has revolutionized analyses of epithelial morphogenesis during development.
- The use of genetic models such as *C. elegans,* Drosophila, zebrafish, and mouse, in combination with the analyses of cells in tissue culture, has enabled the identification of key components of epithelial architecture as well as of regulators of epithelial morphogenesis.
- Defects in epithelial architecture or regulation underlie many human developmental abnormalities and diseases, including cancer.

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GLOSSARY

Cell polarity
A property of cells that have one or more axes of symmetry. In epithelial cells, the polarity is orthogonal to the plane of the cell layer (i.e., apical to basal). By contrast, migrating cells (e.g., mesenchymal cells) are polarized from the leading (front) to the lagging (rear) ends.

Epithelium
A layer of polarized cells that are held together with cell-to-cell adhesions and that are bounded basally by a specialized extracellular matrix called the *basal lamina*.

Extracellular matrix (ECM)
Material that is outside of the cell and that is organized into a meshwork. The ECM is mainly made out of glycoprotein, collagen, and other proteins such as laminin, fibrin, and elastin. One or more of these interact with transmembrane proteins such as integrin, which reside in the epithelial cells. The ECM serves to support and anchor cells, and it regulates intercellular communication.

Morphogenesis
Literally, "the origin of form." During embryogenesis, it refers to the formation of complex structures, including tissues and organs, from initially simple and relatively undifferentiated cells or sheets of cells. Morphogenesis is achieved through regulated cell shape change and migration, which are sometimes accompanied by cell proliferation and/or programmed cell death.

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21 BRANCHING MORPHOGENESIS OF MAMMALIAN EPITHELIA

JAMIE DAVIES

Centre for Integrative Physiology, University of Edinburgh, Scotland, UK

INTRODUCTION

The influences exerted by natural selection on the architecture of organisms vary greatly across different species and habitats, but some evolutionary pressures are almost universal. One is the tendency to maximize the capacity to exchange substances (e.g., oxygen, water, food, or waste) with the environment, because the capacity for such exchange is a serious limit on the rate of an organism's metabolism. Another is the tendency to maximize the efficiency of the internal transport of the same resources. Both problems can be solved by organizing the exchange and transport systems of the organism as approximately fractal (scale-free) structures (Mandelbrot, 1997). Indeed, by assuming that fractal structures dominate exchange and transport, one can derive the well-known "scaling laws" by which measures such as metabolic rate and heartbeat vary with body mass within a type of organism (West et al., 1997; 1999).

One of the most common fractal structures, which was effectively identified as such by Leonardo da Vinci (Long, 2004), is a branching network in which tube diameter and internode length decrease by a constant proportion as branching progresses. It is likely for this reason that such branching networks are a very common feature of multicellular animals, particularly large ones. In humans, for example, internal transport is achieved mainly through an approximately fractal branching system of blood vessels, whereas the communication of gases and waste products proceeds via approximately fractal branched air tubes and urine-collecting ducts. Exocrine organs, such as the pancreas and the salivary and mammary glands, also use branched ductal systems. The absorptive epithelium of the alimentary canal is a rare exception to this rule; it is essentially an unbranched structure, probably because it has to pass solids and because any thinning of lumen diameter would risk blockage. However, its surface is still reminiscent of fractal geometry, consisting of microvilli on villi on ridges.

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Branched structures, then, are essential to the body plan of all but the simplest animals, and their development is an important feature of metazoan organogenesis (Davies, 2005a).

I. TYPES AND SCALES OF BRANCHING MORPHOGENESIS

Branching morphogenesis of animals takes place across a huge range scales, varying from subcellular structures such as dendritic trees to branched cells such as astrocytes to small cell collectives such as Drosophila tracheae to very large multicellular tissues such as the lungs of a blue whale.

The production of branched structures by single cells is outside the scope of this chapter, but a discussion of the relevant subcellular mechanisms can be found elsewhere (Davies, 2005b). The production of multicellular branched structures takes place by four general mechanisms: confluence, intussusception, clefting, and sprouting (Figure 21.1). Although it results in the formation of a branched structure, confluence is not strictly "branching morphogenesis," because nothing actually branches; rather, individual tubules meet and fuse to form a branched system. The development of the pronephric kidney in *Xenopus laevis* proceeds by a mechanism that is very much like that shown in Figure 21.1, A (Vize et al., 2003), and confluence is also important in the production of capillary networks by vasculogenesis (Drake, 2003).

Intussusception is a process whereby a large-diameter tube is split into multiple small-diameter tubes with a similar total cross-sectional area in much the same way that a large river might split into deltas. It is the only one of these mechanisms that can create a branched network from a tube while fluid

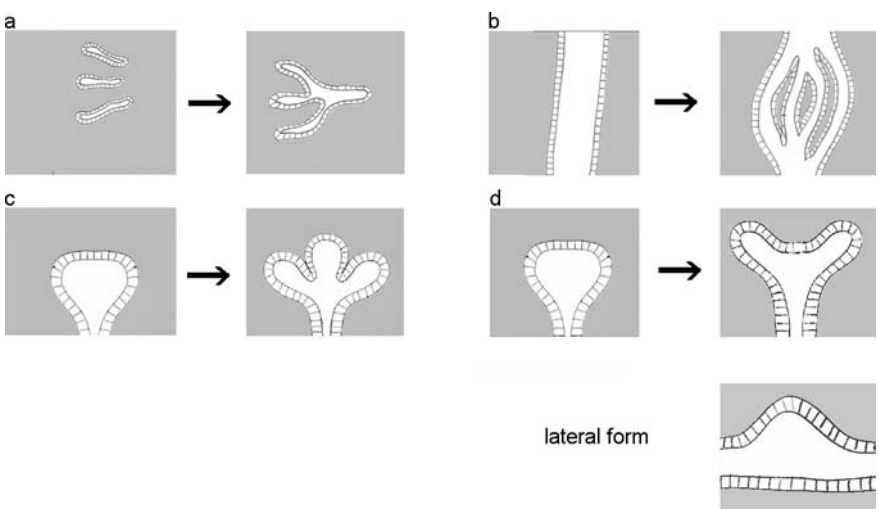

FIGURE 21.1 **The different forms of branching. A,** In confluence, independent tubules flow together to create a branched system. **B,** In intussusception, the walls of the tube fold inward and fuse to form elongated pillars that divide a flow into multiple branches. **C,** In clefting, deep clefts divide the end bud of an epithelial tubule into different tips. **D,** In sprouting, new tips sprout outward from either the end (top diagram) or the side (bottom diagram) of an existing tubule.

continues to flow, and it is therefore very important in the development and remodeling of the vascular system (Patan, 2004). Its mechanisms are so different from clefting and sprouting that it will not be considered further in this chapter (see Chapter 33).

Clefting and sprouting are responsible for the majority of branched epithelial systems, and the rest of this chapter will concentrate on them. There are two basic ways in which epithelial buds produce a branched system: monopodial branching and dipodial branching. In monopodial branching, a principal "trunk" extends forward, and lateral branches form from its sides (Figure 21.2); a classical Christmas tree grows by monopodial branching. In dipodial branching, the bud bifurcates to form two equal branches, which then bifurcate again, and so on (see Figure 21.2); many seaweeds grow by dipodial branching. Sometimes branching can be by trifurcation rather than bifurcation, and organs such as the kidney show a mixture of both bifurcation and trifurcation (Watanabe and Costantini, 2004). The epithelial tubules of some organs can use both methods in sequence. For example, mammary glands use dipodial branching to create the tree of milk ducts and then use a form of monopodial branching to create groups of alveoli along them (Fata et al., 2004).

Monopodial branching always occurs by a process of sprouting in which part of the wall of an existing duct bulges outward and becomes the growing tip of a new duct (see Figure 21.1, D). Many examples of dipodial branching also appear to take place by sprouting. In the ureteric bud of the kidney, for example, movies made of branching morphogenesis using green-fluorescent-protein–tagged tissues (Watanabe and Costantini, 2004) clearly show the outward bulging of new branches, with little evidence of clefts. In other organs, such as the salivary gland, the broad ends of the branching tubules (sometimes called "ampullae" because of their shape) seem to be split by narrow clefts (see Figure 21.1, C; Nakanishi and Ishii, 1989; Miyazaki, 1990; Nakanishi et al., 1987). It is possible that these clefts represent a stationary restriction around which the expanding epithelium has to divide (Nakanishi et al., 1988; described later).

In the branching epithelia of mammals, ampullae and the sprouts that arise from them seem to always be composed of a population of cells (as shown in the diagram below) rather than beginning as processes from one single cell; this is in marked contrast with the tracheal system of *Drosophila melanogaster*, which is the most closely studied invertebrate branching epithelium.

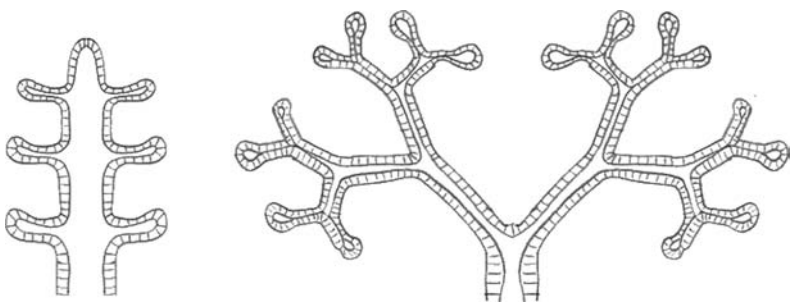

FIGURE 21.2 Monopodial branching, in which lateral branches are emitted from a principal "trunk," contrasted with dipodial branching, in which a tree is formed by successive divisions of branch tips.

II. THE ROLE OF GENETICS IN STUDYING MECHANISMS OF BRANCHING MORPHOGENESIS

Genetics is used in the study of branching morphogenesis in much the same way that it is used in other developing systems. Sporadic mutants that generate a phenotype with aberrant branching of some or all arborizing epithelia identify genes that play an essential role in normal branching morphogenesis. Also, precisely induced mutations in transgenic animals can be used to test specific hypotheses about the involvement of particular molecules by a reverse-genetic approach. Genetics is but one approach to the problem, however, and a variety of pharmacological and culture techniques are also used, because these have the power to reach cellular processes that do not depend on changes in gene expression. Cellular signal transduction systems are examples of such processes.

Classical and reverse genetics have identified a vast range of mutations that have an effect on branching. Attempting to describe them all here would not be particularly informative. Instead, this chapter will consider the principal mechanisms of branching morphogenesis and the genes that are involved without attempting to be comprehensive. The mechanisms will be considered in an order that proceeds from the molecular level to the system level.

III. CELLULAR MECHANISMS OF BRANCHING MORPHOGENESIS

One of the least-understood aspects of branching morphogenesis is the connection between the activity of genes and the production of actual morphologic change. The sprouting of new branches either from the side or from the ampulla of an existing tubule requires some mechanism that results in the local advancement of a group of cells. In principle, this could be achieved by change in those epithelial cells to make them more inclined to bulge out, by change in the surrounding mesenchyme to reduce its mechanical resistance, or by a combination of the two.

The basal surfaces of most epithelia that branch *in vivo* or in organ culture appear to be typical of epithelial basement membranes (Meyer et al., 2004), and the outline of the cells is smooth. This suggests that no invadopodia are involved in the forward movement of the cells (Meyer et al., 2004) and that the sprouting of new branches is therefore by a process that is quite distinct from the invasive activity of a metastatic carcinoma, in which cells tend to advance as individuals. Cells that form a new sprout of a branching epithelium thus do so as a coordinated group. One of the ways in which an epithelium can bend outward is by cell deformation so that each previously columnar cell becomes wedge shaped. When this occurs across a population of cells within an epithelium, the result is an outpushing of a bulge of cells (Figure 21.3). One way in which this cell deformation can be achieved is by the constriction of the microfilaments that run across and around the apical end of the cell (where most adhaerens junctions are located). In the ureteric bud of the kidney, there is evidence that such a system might operate. Actin is expressed intensely at the apical ends of cells that are participating in bulge formation, and interfering with the formation of actin or interfering with the ability of myosin to exert tension on actin inhibits branching (Michael et al., 2005). Furthermore, pharmacological interference with the kinase ROCK,

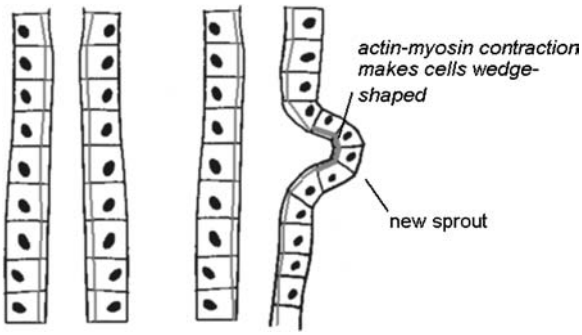

FIGURE 21.3 **The production of a new sprout by localized actin–myosin tension driving cell wedging.** (See color insert.)

through which the small GTPase Rho controls myosin activity, produces the same effect (Michael et al., 2005). Similar experiments have yielded similar results in developing lungs (Goldin et al., 1984; Moore et al., 2005). Together, these observations suggest that actin–myosin contraction is important to the production of new branches. However, they do not confirm that the mechanism is cell wedging: for example, it could be that the strong actin contraction everywhere *except* in the location of the future bulge forces the expanding epithelium to push out at that point.

Branching epithelia are surrounded beyond their basement membranes by a mesenchymal extracellular matrix through which any new sprout has to pass. A combination of genetic and biochemical evidence has implicated matrix metalloproteinases (MMPs) in the process of matrix clearance (Sympson et al., 1994; Lee et al., 2001; Lelongt et al., 1997; Witty et al., 1995). Matrix metalloproteinases are a family of matrix-degrading molecules, each with a distinct specificity, that are secreted into the matrix and that are themselves activated by proteolysis (Mott and Werb, 2004). In principle, locally elevated destruction of the matrix might encourage the emergence of an epithelial sprout without any special morphogenetic activity in the epithelial tissue itself. Indeed, there is evidence that a membrane-bound MMP called MT1-MMP, which is involved in the activation of other diffusible MMPs, is expressed preferentially by cells that are involved in branching (Meyer et al., 2004; Ota et al., 1998). Reducing the expression of MT-MMP1 with antisense oligonucleotides reduces ureteric bud branching in organ culture (Kanwar et al., 1999). Blocking the activity of MMP9 using antibodies can block branching morphogenesis in cultured kidneys (Lelongt et al., 1997), and a variety of MMP-inhibiting drugs strongly inhibit the branching of isolated ureteric buds grown in three-dimensional collagen matrices (Pohl et al., 2000). However, kidneys of transgenic mice that lack MMP9 develop normally in the whole embryo (Andrews et al., 2000), suggesting perhaps that there is redundancy in that context.

The expression of certain MMPs is enhanced by branch-promoting growth factors in both the lungs and the mammary glands; furthermore, the lungs of MMP2 knockout animals show abnormal morphogenesis of the airways (Werb, 1997). Expressing autoactivating MMP3 in the mammary gland can greatly enhance the branching of milk ducts and even induce the precocious production of milk-secreting alveoli (Sympson et al., 1994; Wiseman

et al., 2003). In organoid culture, exogenous MMP3 can even drive branching in the absence of growth factors that are normally required for it to take place (Simian et al., 2001). However, because added MMP3 activity was distributed globally in both the transgenic mice and the organoid cultures, it is difficult to see how it might have acted directly to encourage sprouting, and it is likely that its action may therefore have been indirect (e.g., by liberating growth factors or creating bioactive matrix fragments). Interpretation becomes even more complicated when it is recognized that MMPs may also destroy branch-promoting molecules in the matrix. For example, tenascin promotes airway branching in the lung. When lungs develop in hypoxic conditions, they reduce their production of MMPs and therefore allow more tenascin to accumulate; this apparently promotes more branching (Gebb and Jones, 2003; Hosford et al., 2004).

Local accumulation of extracellular matrix molecules is thought to be responsible for clefting. In the salivary gland, for example, the cleft is full of thick fibers of collagens, particularly type III collagen (Nakanishi et al., 1988; Fukuda et al., 1988). Collagen fibers can bear considerable tension (e.g., they are a major component of ligaments), and, in one model for branching, they function to hold back the local advance of the epithelium and thus force it to divide into two separate tips. This model is supported by the observations that reducing the amount of collagen using exogenous collagenases decreases clefting, whereas inhibiting endogenous collagenase activity enhances clefting (Hayakawa et al., 1992; Spooner and Wessells, 1970).

The collagens of the cleft form on a scaffold of fibronectin (Jiang et al., 2000), and inhibiting fibronectin synthesis using RNAi or inhibiting fibronectin function using antibodies blocks the formation of the bundles and therefore blocks the formation of clefts (Hieda et al., 1996; Sakai et al., 2003). The fibronectin scaffold is itself organized by integrins expressed by the epithelia. It is interesting to note that fibronectin synthesis is also required by branching epithelia such as those of the kidney (Sakai et al., 2003), which are not generally considered to use clefting. It is therefore possible that the two mechanisms of branching—sprouting and clefting—are more similar than their morphologies imply.

IV. CONTROL OF BRANCHING ACTIVITIES BY INTRACELLULAR SIGNAL TRANSDUCTION SYSTEMS

Experiments using both pharmacological and genetic techniques have identified certain intracellular signaling pathways as being critical to branching morphogenesis in a number of systems. The Erk mitogen-activated protein kinase (MAPK) pathway (Figure 21.4) is required for branching in a number of systems. Genetic evidence shows it to be required for tracheal branching in *D. melanogaster*, and pharmacologic evidence shows it to be necessary in the mammalian kidney, salivary gland, mammary gland, and lung (Kling et al., 2002; Kashimata et al., 2000; Niemann et al., 1998; Fisher et al., 2001; Hida et al., 2002; Tefft et al., 2005). The PI-3-kinase pathway (Figure 21.5) is also required for branching in the kidney, lung, salivary gland, and mammary gland (Cantley et al., 1994; Niemann, Brinkmann, Spitzer, et al. 1998; Karihaloo et al., 2001; Liu et al., 2004; Larsen et al., 2003; Tang et al., 2002; Derman et al., 1995).

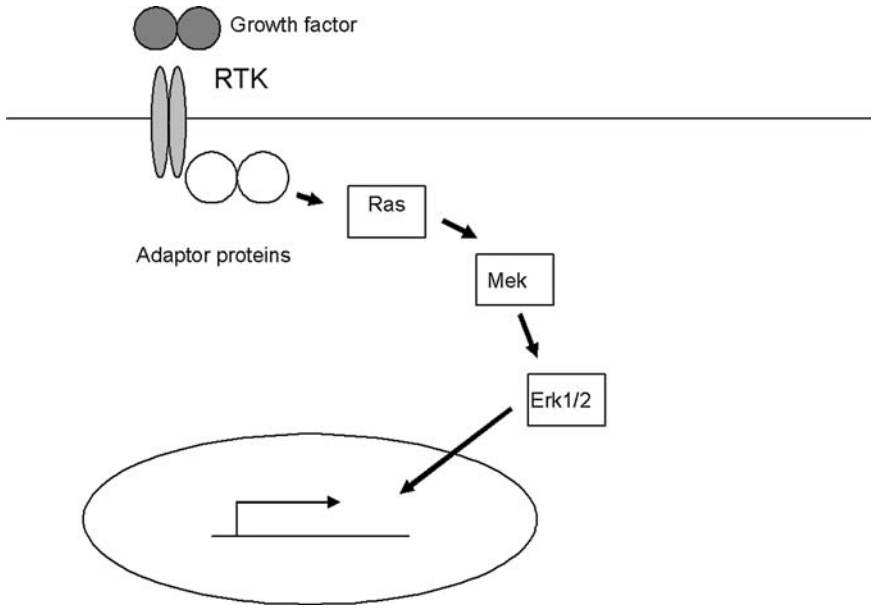

FIGURE 21.4 **The Erk MAPK pathway.** (Heldin and Purton, 1996).

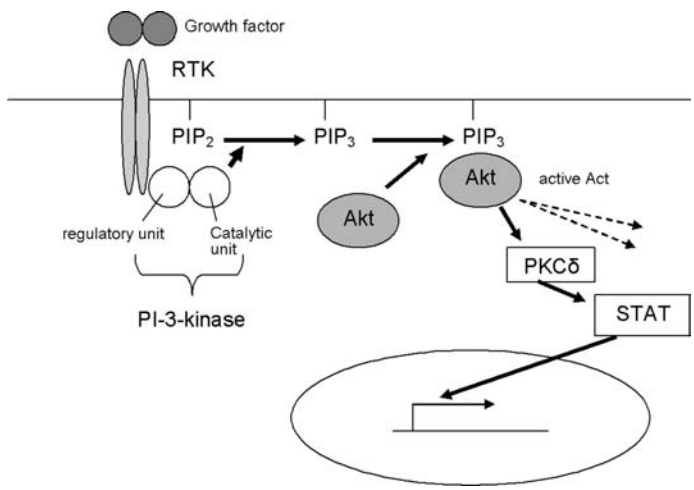

FIGURE 21.5 **The PI-3-kinase pathway.**

The Smad pathway (Figure 21.6) can act as an activator or as an inhibitor of epithelial branching (Davies, 2002), but the role of the pathway seems to be less conserved among organs than is the case for Erk and PI-3-kinase. In lungs, the activation of R-Smad1 encourages branching, whereas the overexpression of Smurf1 (which ubiquitinates R-Smad1 and therefore targets it for destruction) or the antisense knockdown of R-Smad1 inhibits branching

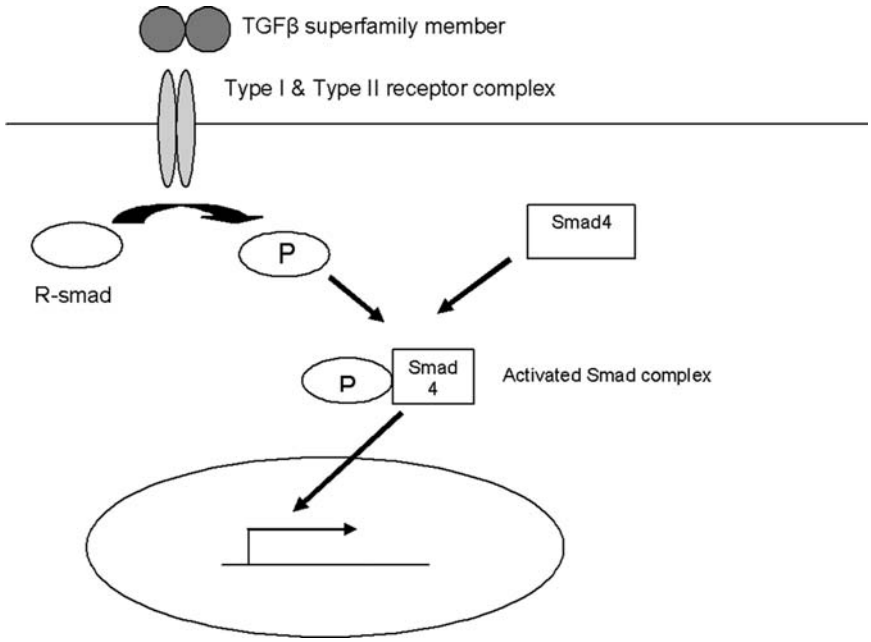

FIGURE 21.6 **The Smad signalling pathway.** (Massague et al., 2005.)

(Shi et al., 2004; Chen, et al., 2005). Alternatively, in kidneys, the activation of R-Smad1 inhibits branching, and dominant-negative R-Smad1 encourages it (Piscione et al., 2001; Hartwig et al., 2005). In lungs, R-Smad2 inhibits branching, whereas the antisense inhibition of either R-Smad2 and R-Smad3 or R-Smad4 encourages branching (Zhao et al., 2000; Zhao et al., 1998).

The Wnt signaling pathways (Figure 21.7) also play a role in epithelial branching. This is known mainly from the effects of Wnts and their natural extracellular inhibitors on branching morphogenesis (described later), but a little data have been gathered about the signaling pathways themselves. The

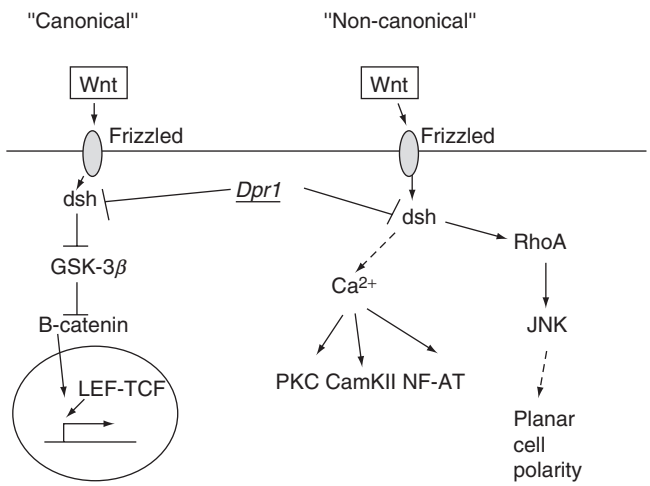

FIGURE 21.7 **The different intracellular pathways through which Wnts can signal inside cells.** (Adapted from Pandur et al., 2002, with additional data from Sheldahl et al., 2003.)

TOPGAL reporter mouse, which activates β-galactosidase expression in response to the canonical Wnt signaling pathway, shows this pathway to be active in the branching epithelium of the lung (De Langhe et al., 2005). However, in both the lung and the salivary gland, this Wnt pathway seems to inhibit branching. Stimulating it using exogenous Wnt3a or lithium ions (which inactivate GSK3β; Klein and Melton, 1996) inhibits branching, whereas blocking it using β-catenin morpholinos stimulates branching (Dean et al., 2005). In mammary glands, the overexpression of Axin, which would downregulate canonical signaling, does not block branching (Hsu et al., 2001), thereby suggesting that in this system, too, canonical Wnt signaling is inhibitory or at least just neutral.

Blocking Wnt signaling in general using an excess of natural Wnt antagonists (DKK1 in mammary gland experiments, sFRP1 in kidney experiments) inhibits branching morphogenesis. When this fact is put together with the observation that canonical Wnt signaling inhibits branching, the most obvious implication is that branching is stimulated by the noncanonical pathways and that their effects dominate. There is little direct evidence for these pathways, although we have recently obtained pharmacologic evidence that Jnk, which is a critical component of the planar cell polarity pathway, is important to branching in kidneys.

It is not yet clear how the pathways discussed in this section link to the Rho–ROCK–myosin–actin pathway described in the previous section. In other systems, pathways have been discovered that link both Erk MAPK and PI-3-kinase pathways to Rho. The protein ARAP3, for example, is activated by the $PI(3,4,5)P_3$ product of PI-3-kinase, and it is a GTPase-activating protein for Rho (Krugmann et al., 2004). Rho activity can also be controlled by Erk activity, albeit by a rather indirect route (Vial et al., 2003). It is also clear that, in other systems, PI-3-K and Erk activities can be controlled *by* Rho (Reuveny et al., 2004; Liu et al., 2004), so untangling the connections among these various pathways in branching epithelia will not be trivial.

V. CONTROL OF BRANCHING ACTIVITY BY EXTERNAL SIGNALS

The activity of the branch-promoting and branch-inhibiting pathways outlined previously is controlled by extracellular signals, most of which arise from the mesenchyme cells that surround each branching epithelium. Genetics has proved to be a powerful method for identifying these signals; indeed, the similarity of the phenotype of two mutations has often been the first hint that a particular growth factor is the ligand for an "orphan" receptor.

The Erk MAPK and PI-3-kinase pathways are activated by a number of receptor tyrosine kinases (RTKs). This allows a greater choice of RTK to achieve essentially the same purpose; different organs do use different RTKs and different extracellular ligands to control branching in similar ways (Davies, 2002). The branching epithelia of many organs (lungs, pancreas, salivary glands, mammary glands, prostate, and kidney) express the fibroblast growth factor receptor (FGFR)-IIIb RTK. The lungs, pancreas, and salivary glands use fibroblast growth factor (FGF)-10 as the main ligand to act on FGFR-IIIb, whereas the mammary glands and prostate use FGF-7. The kidneys use FGF-7 as well, but in that organ the whole FGF system assumes relatively minor importance, and the Ret RTK and its

ligand GDNF are the major stimulators of branching. In addition, all of these organs express epidermal growth factor receptor (EGFR), and branching is stimulated by its ligands epidermal growth factor (EGF) and/or transforming growth factor (TGF)-α. Most also express the Met RTK, and branching and/or elongation is stimulated by its ligand HGF. In addition to these ligand-receptor complexes, other ligand-receptor complexes are used by various organs. These include amphiregulin, neuregulin, EGF, TGF-α, colony stimulating factor, and insulin-like growth factor (Wang and Laurie, 2004).

In addition to having receptors for stimulators of branching, cells of branching epithelia also have receptors for inhibitors of branching, which are also produced by surrounding mesenchyme. Many of these inhibitors are members of the TGF-β superfamily. The lungs, mammary glands, and kidneys bear receptors for TGF-β itself, which signals via R-Smad2 and R-Smad3 (Bartram and Speer, 2004) and which acts as a powerful inhibitor of branching (Bartram and Speer, 2004; Ritvos et al., 1995). Branching in the pancreas, salivary gland, mammary gland, salivary gland, and prostate is also inhibited by activin, which also signals via R-Smad2 and R-Smad3. Bone morphogenetic proteins (BMPs), which signal via R-Smad1, are inhibitory in some systems. In the kidney, for example, BMP-2 acts as a powerful inhibitor of branching, whereas BMP-7 acts as a stimulant at low concentrations and an inhibitor at high concentrations; the inhibitory action depends on R-Smad1 (Piscione et al., 2001), whereas the stimulatory effect proceeds via p38 MAPK (Hu et al., 2004).

Various Wnt proteins, which drive the Wnt pathways outlined in Figure 21.7, are expressed by epithelia and by their surrounding mesenchymes in different organs. Some organs express few Wnts. For example, the developing prostate seems to express just Wnt4 (Zhang et al., 2006). Lungs express Wnt2 in the mesenchyme, Wnt7b in the epithelium, and Wnt11 in both; some Wnt knockouts result in branching abnormalities (Pongracz and Stockley, 2006). In the kidneys, the branching epithelium expresses Wnt9b and Wnt11, whereas the mesenchyme expresses Wnt4 and Wnt7b in different places (Carroll et al., 2005; Stark et al., 1994; Gavin et al., 1990; Kispert et al., 1996; also described later). Mammary glands express Wnt2, Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7b, and Wnt10b in complex patterns during their development (Lane and Leder, 1997; Weber-Hall et al., 1994; Bradbury et al., 1995). Genetic and culture experiments have shown that Wnt1 and Wnt4 both encourage branching morphogenesis in the mammary glands.

These are not the only ligands that control branching morphogenesis, and the roles of other families (e.g., Notch) are currently the subject of close attention. However, it should be noted that conventional genetics has not proved to be as useful for finding molecules that control branching as might be hoped; perhaps because of redundancy in a living embryo, the effects of knocking out a gene *in vivo* tend to be much less than those of inhibiting gene function in culture (Davies, 2002).

VI. BRANCHING MORPHOGENESIS IS A LOCAL RATHER THAN A GLOBAL ACTIVITY

One of the surprises of recent research has been the finding that cells of branching epithelia are not all in the same state of differentiation and that some are specialized for initiating new branches. This was a surprise, because

two early lines of evidence (one theoretical and one experimental) suggested that a homogenous population of cells would be able to branch.

The theoretical basis for this idea came from the study of the physical phenomenon of viscous fingering. Viscous fingering takes place when one fluid (e.g., air) is pumped under pressure into a more viscous fluid (e.g., oil between two coverslips). The formation of branches happens because the rate of advance of any part of the interface between the two fluids is proportional to the local pressure gradient; this is higher at the tips of thinner processes. Therefore, a smooth front will break spontaneously into "fingers." These fingers are prevented from becoming arbitrarily fine by the fact that sharply bent interfaces are energetically unfavorable for reasons of surface tension, and the dimensions of viscous fingers are thus defined by a compromise between the tendency of the pressure gradients to create ever-finer processes and the tendency of surface tension to flatten them out. More detailed explanations of this phenomenon can be found elsewhere (Fleury et al., 2004; Davies, 2005b). The key point here is that, if a low-viscosity population of cells was to increase in volume (perhaps by multiplying) in a higher-viscosity matrix, branching morphogenesis would be expected to take place by physics alone; there is no need for "leading" cells to be specialized in any way.

The early experimental evidence for a homogenous population came from a culture model for epithelial branching that is quite similar to the viscous fingering example described previously. In it, immortalized cells derived from branched epithelia (e.g., renal collecting ducts) are suspended in three-dimensional collagen matrices and provided with suitable growth factors. Over a few days, the cells form cysts that then send out spikes and branching tubules in a process that adherents of this system allege is a close analogue of branching morphogenesis *in vivo* (Figure 21.8; Santos and Nigam, 1993; Balkovetz, 1998). The cells in these systems appear to share the same state of differentiation (although this question has not been examined rigorously), but branching still takes place.

The examination of patterns of gene expression in real branching epithelia has, however, revealed that at least two types of cells are present. One is found only at the tips of branches, and the other is found in the remainder

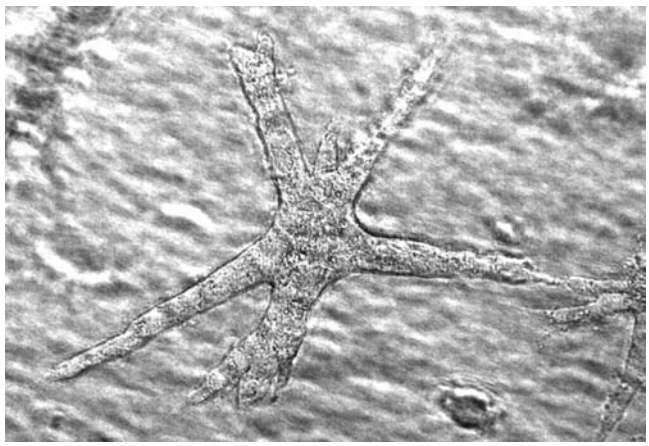

FIGURE 21.8 **Branching of a group of mIMCD3 renal epithelial cells grown in a three-dimensional collagen matrix in the presence of the branch-promoting growth factor HGF.** (Photo courtesy of Nils Lindstrom of the University of Edinburgh.)

of the branching system (the "stalks"). The cells differ in the expression of a number of key genes, many of which are connected with signaling. Examples are shown in Table 21.1; many more have been identified using microarrays (Lu et al., 2004; Schmidt-Ott et al., 2005).

The specialized gene expression of tip cells is reflected in their cell biology. For example, in mammalian systems in which cell proliferation has been studied, it is found to take place mainly in the branch tips (Michael and Davies, 2004; Goldin and Opperman, 1980). This observation gives rise to a model of development in which cells multiply mainly at the tips, with some daughter cells remaining in the tips and others being "left behind" by the advancing tip to form new regions of the stalk (Figure 21.9). The combination of self-renewal and differentiation implied by this model further suggests that the branch tips may harbor stem cells. Careful study of the mammary gland has indeed

TABLE 21.1 Evidence from Gene Expression Studies in a Number of Branching Epithelia that Tips and Stalks are in Distinct States of Differentiation

Organ	Gene	Tip/Stalk	Reference
Kidney	Wnt11	Tip	Kispert et al., 1996
	Sox9	Tip	Kent et al., 1996
	Collagen XVIII	Stalk	Lin et al., 2001
Lung	BMP4	Tip	Li et al., 2005
	Wnt7b	Tip	
	Spry2	Tip	Hashimoto et al., 2002
	Shh	Tip	Li et al., 2005
	Notch	Tip	Post et al., 2000
	Netrin1	Stalk	Liu et al., 2004
	SP-C	Tip	Hashimoto et al., 2002
Prostate	BMP7	Tip	Grishina et al., 2005
	Shh	Tip	Pu et al., 2004
Pancreas	EphB2, B3, B4	Tip	van Eyll et al., 2006
	Ngn3	Stalk	van Eyll et al., 2006
Salivary gland	Smo	Tip	Jaskoll et al., 2004
	Gli3	Stalk	Jaskoll et al., 2004

This table lists representative examples of differentially expressed genes; it is not intended to be a complete list.

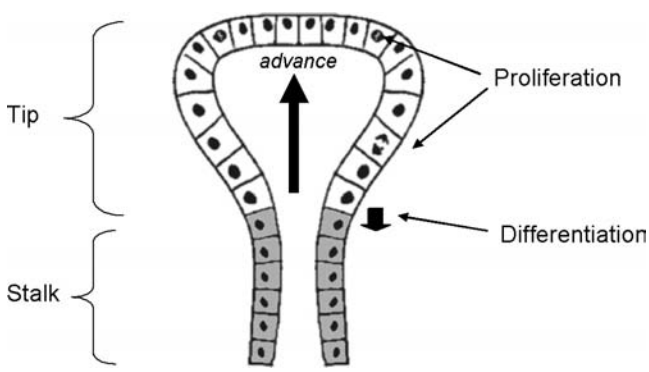

FIGURE 21.9 Proliferation takes place mainly in the tip regions. Cells "left behind" by the advancing tip (perhaps passively, perhaps by their own inactivity) differentiate into stalks.

identified a population of slowly cycling cells that have the properties of stem cells (Kenney et al., 2001) and that can form a complete mammary tree if transplanted into the empty fat pad of a host mouse (Smith, 1996; Shackleton et al., 2006). However, they are distributed in both the tips and the stalks (Kenney et al., 2001; Smith, 2005). This location might allow for the formation of lateral branches later; we have found that, in the kidney, cut stalks have the ability to regenerate tips; it may be that here, too, stem cells left behind in the stalk are responsible.

If most branching activity takes place in the specialized tips (rather than being a global increase in pressure, as it would be for viscous fingering), the tissue must have some mechanism for deciding which cells should be tips, which should be stalks, and when tips should branch. Results obtained during the last decade or so strongly imply that these decisions are made by a control system using closed-loop feedback (Schwarzenbach, 1996).

VII. THE ROLE OF FEEDBACK IN SHAPING THE BRANCHING TREE

There seem to be two types of feedback involved in spacing out branches of a growing epithelial arbor: those that operate within the epithelium itself and those that operate via the mesenchyme.

One of the first examples of intraepithelial feedback in a branching system was discovered in the tracheae of *D. melanogaster*. These branch within the tissues, but they are not directly comparable with mammalian systems, because they involve very few cells, and each branch tends to be led by one specific cell. However, they do use an FGF-FGFR-MAPK branch-promoting signal that is similar to the one used in mammalian lungs. The tip cells, in which MAPK is active, produce a protein, Sprouty, that inhibits MAPK signaling to a level that produces only the required amount of branching (the name of the protein arises from the corresponding mutant phenotype, in which the tips form far too many sprouts).

Mammals have several homologues of Sprouty (Guy et al., 2003). In the lungs, Sprouty2 is made by cells at the tip of the branching airways, and this inhibits MAPK signaling (Mailleux et al., 2001). Its production is driven by MAPK activity itself in a positive feedback loop that acts to restrict MAPK more strongly as MAPK activity rises (Ozaki et al., 2001) and thus prevents excess sprouting. The branching epithelia of kidneys also express Sprouty2, and it seems to operate in much the same manner. The "setpoint" of MAPK activity, which maintained by Sprouty, is presumably set so that there is only just enough branch-promoting activity to allow for one branch event per tip at a time. This might reflect a very delicate balance, and the low (but non-zero) frequency of trifurcations seen in renal branching events perhaps reflects the presence of the occasional failure of Sprouty2 to clamp MAPK activity down quite hard enough.

The operation of a mesenchyme-borne feedback system is demonstrated very well in the developing kidney, in which the developmental biology of the mesenchyme has been studied rather more than in other organs (because the mesenchyme is the ultimate source of most of the clinically important parts of the kidney). Mesenchyme that has not yet been penetrated by the branching epithelium is a potent source of GDNF, which is the main stimulant of branching in this organ (described previously). After a particular area of

mesenchyme is penetrated by branching epithelium, however, signals from the epithelium (Wnt9b [Carroll et al., 2005] and perhaps others) induce the mesenchyme to change its state of differentiation. The mesenchyme undergoes a complex series of developmental changes that are beyond the scope of this chapter (see Chapter 35) and that result in the production of epithelial nephrons and mature renal stroma. Critically, it ceases to produce branch-promoting GDNF, and it begins to produce branch-inhibiting molecules such as BMP2 and, eventually, TGF-β. Thus, the system is arranged so that the virgin mesenchyme ahead of branching epithelium encourages the production of new branches (and probably also attracts them), but any areas that have already been penetrated inhibit any further branching activity. This ensures that branching is not excessive, and it tends to maintain a constant ratio of branches per unit volume of mesenchyme.

In the lungs, the main stimulant of branching is FGF-10. The epithelium produces Shh, which does not diffuse far but which inhibits the mesenchymal production of FGF-10 (Bellusci et al., 1997; Miller et al., 2001). Again, the effect is to limit the expansion of the branching epithelium to virgin mesenchyme.

The use of feedback mechanisms such as these makes branching systems tolerant of errors. If an area of mesenchyme is "missed" the first time around, it will remain attractive, and it may therefore promote a lateral branch and thus eventually be served by the branching system. Indeed, this is probably the reason for the lateral branching that is observed—albeit infrequently—in developing kidneys (Srinivas et al., 1999).

Feedback can go beyond the simple tolerance of errors, however; it makes branching systems effectively self-organizing, and it therefore makes them qualitatively different from many of the other aspects of developmental biology studied by geneticists. In wild-type animals, the number and exact location of such aspects of the body plan as fingers, vertebrae, and teeth are constant and predictable. The number and exact location of epithelial branches in an organ are far less reproducible and predictable, however, even in left–right "duplicate" organs of the same animal. The same is true of microvasculature. This occurs because the precise arrangement of the branches is not specified in the genetic program; rather, that program specifies a system of feedback that can organize branching tissues into patterns that are statistically predictable but not predictable in precise detail. This method of embryonic development—adaptive self organization—is discussed in detail elsewhere (Davies, 2005b).

Branching morphogenesis, then, is a complex process that is regulated by genetic and other controls that operate at a variety of nested levels. This chapter, along with most of the research performed during the last few decades, has emphasized common themes that can be found in most or all branching epithelia of mammalian organs. However, there are differences, and it is likely that future research will emphasize these differences, because they may explain why a mammary gland, for example, does not look like a lung.

SUMMARY

- Branching morphogenesis of epithelia is a common event in mammalian organogenesis.
- Branching patterns vary; the types of patterns are monopodial, dipodial, budding, clefting, confluence, and intussusception.

- Branching morphogenesis is driven by cytoskeletal contraction, matrix modification, and proliferation.
- Branching morphogenesis is regulated by extracellular growth factors, particularly FGFs and BMPs.
- Regulatory growth factors tend to act in feedback loops so that the architecture of the branched system arises by adaptive self-organization.

GLOSSARY

Bifurcation
The splitting of the tips of a branch into two equal new tips.

Confluence
The running together and fusion of initially separate tubules to produce a branched system.

Dipodial branching
Producing new branches by the division of the tips of existing branches, which is in contrast with monopodial branching.

Intussusception
Longitudinal division of a tube (by local fusion of its opposite walls) into a number of finer tubes.

Monopodial branching
Producing new branches from a central "trunk."

Self-organization
The ability of a collection of autonomous agents (e.g., cells) to organize themselves according to internal rules rather than external control. If the process allows the organization to vary in response to its environment, the term *adaptive self-organization* is used.

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RECOMMENDED RESOURCES

Affolter M, Bellusci S, Itoh N, et al: Tube or not tube: remodeling epithelial tissues by branching morphogenesis, *Dev Cell* 4:11–18, 2003.

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22 THE ROLES OF EPHRIN–EPH IN MORPHOGENESIS

IRA O. DAAR

Laboratory of Cell and Developmental Signaling, National Cancer Institute–Frederick, Frederick, MD

INTRODUCTION

The creation and organization of the embryonic body into distinct tissues requires the coordinated behavior of cells through adhesive mechanisms that promote and maintain the segregation and sorting of different cell populations. Controlling the movement and sorting of cells is the underpinning of morphogenesis that results from the alterations to the cytoskeleton, the control of cell division, and gene transcription. Regulating the adhesive and migratory properties of cells is critical to forming, organizing, and maintaining tissue patterns during development. In this chapter, we will attempt to survey and give some perspective on the role of the Eph family of receptor tyrosine kinases and their ligands, the ephrins, in morphogenetic processes. These receptors and ligands are expressed in a dynamic fashion during embryogenesis, and they are found in the ectoderm, mesoderm, and endoderm of vertebrates. A body of evidence is emerging that shows a requirement for these proteins in the proper migration of cells, in the formation of boundaries between structures, and for the control of cell shape. We will discuss the genetic and experimental evidence for the role of signaling from Eph receptors and ligands in various morphogenetic processes during development, and we will also discuss how the deregulation of these molecules can lead to disease as well as represent targets for therapeutic action.

I. CONSERVED EPHRIN/EPH STRUCTURE FROM *CAENORHABDITIS ELEGANS* TO *HOMO SAPIENS*

A. Existence of Two Classes of Receptors and Ligands: Glycosylphosphatidylinositol Linked (A) and Transmembrane (B)

Eph receptors are transmembrane receptor tyrosine kinases that possess an extracellular domain that includes an N-terminal ligand-binding domain, a

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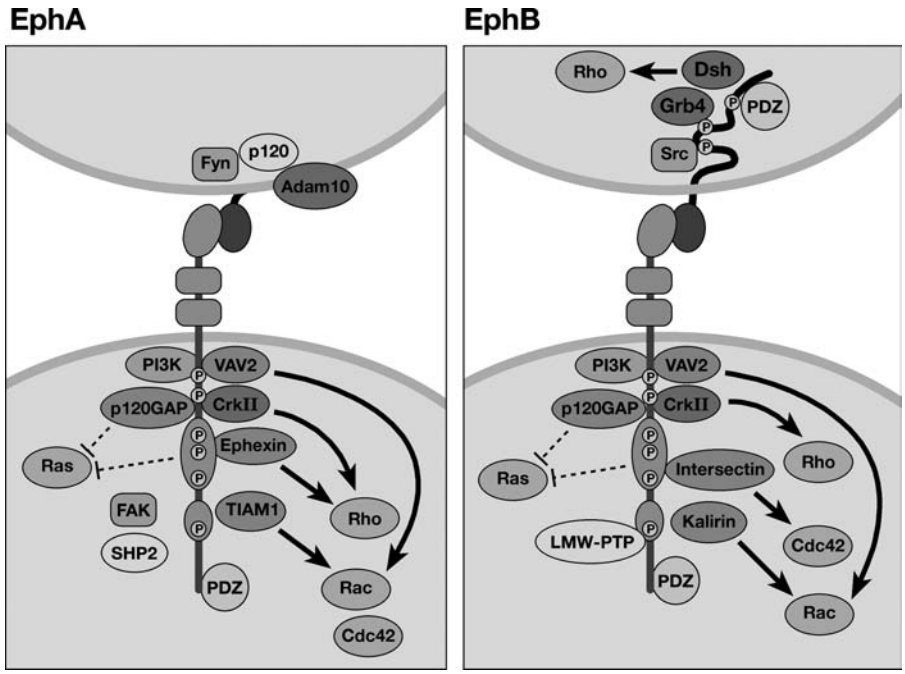

FIGURE 22.1 **Eph–ephrin associated signaling molecules**. The diagram displays some of the signaling molecules associated with A- or B-type Eph receptors and ephrins. *Dark arrows* represent positive influences, whereas *dotted cross bars* represent inhibitory activities.

cysteine-rich epidermal growth factor–like domain, and two fibronectin type III motifs. The intracellular domain contains several tyrosine phosphorylation sites, a single kinase domain, and two protein–protein interaction domains: a sterile α motif and a C-terminal PDZ (post-synaptic density protein PSD-95, discs-large septate junction protein, and the epithelial tight-junction protein ZO-1) binding motif. These receptors are divided into two subclasses (A and B) by sequence similarities and by binding specificity toward two subclasses of ligands (A and B), known as *ephrins*. The ligands are all membrane-bound proteins, with the A subclass being glycosylphosphatidylinositol linked to the membrane and the B subclass being transmembrane proteins that have a short cytoplasmic domain. Generally, the A-type receptors have specificity toward A-type ligands, whereas B types bind to their cognate receptors (Figure 22.1). The exceptions to this rule are EphA4 and EphB2 (Pasquale, 2005; Poliakov et al., 2004).

B. Eph Receptors and Ligand Interactions

The interaction between Eph receptors and ephrins occurs during cell–cell contact events during development. During cell contact, a high-affinity monovalent interaction occurs between the Eph receptor on one cell surface with an ephrin molecule on a juxtaposed surface (Himanen and Nikolov, 2003). The globular ephrin-binding domain of the receptor interacts with a hydrophobic loop of ephrin, and additional binding interfaces on both molecules are also engaged in the process. Low-affinity sites on opposite sides of their respective molecules may contribute to the tetramerization of the eph–ephrin dimers and to specificity of subclass binding (Pasquale, 2005). These tetramers can aggregate into clusters, depending on the density of Eph receptors and ephrins on the cell surfaces.

The lateral expansion of these clusters on the cell surface may broaden the signaling domain beyond the areas of contact on the cell surface.

C. Repulsion and Attraction

Eph receptors and ligands have a bidirectional mode of signaling during which both molecules transmit intracellular signals within their respective host cells (Figures 22.1 and 22.2). Eph–ephrin interactions induce cell repulsive or attractive responses in several cell types, and they may have different effects (repulsion or attraction and adhesion) within a subpopulation of cells (see Figure 22.2). The mechanism that allows repulsion is still unclear, but recent evidence indicates that cells expressing Eph or ephrins are at least competent to repel each other through a loss of the Eph–ephrin adhesive complexes via an endocytic mechanism (see Figure 22.2; Zimmer et al., 2003; Marston et al., 2003). This mechanism allows for the removal of the complexes from the cell surface, and it allows cells to disengage from each other. Alternatively, the release mechanism may be accomplished through a proteolytic cleavage of ephrin by a membrane metalloprotease (ADAM10; see Figure 22.1; Hattori, 2000; Janes et al., 2005).

Adhesion may be promoted by the inhibition of repulsion, thereby allowing the physical binding of Eph and ephrins to cause cell–cell adhesion (see Figure 22.2). However, the signaling that accomplishes this is still unknown. One mechanism may be through naturally occurring forms of Eph receptors that lack kinase activity and thus act in a dominant-negative manner. Another mechanism is the modulation of signaling by alternative receptors and other signaling molecules (Jones et al., 1998; Moore et al., 2004). However, there may also be many other intracellular signaling mechanisms that lead to such a block of repulsion (see Figure 22.2; Pasquale, 2005).

D. Associated Signaling Molecules

What are the intracellular signaling molecules that transmit Eph receptor and ephrin signals? There are a variety of cytoplasmic proteins that regulate the adhesion and organization of the cytoskeleton that have been found to associate with both molecules, but few have been functionally ascribed *in vivo*. It has been over a decade since the concept of reverse signaling through the intracellular domain of transmembrane ephrins was introduced. In one form, this signaling occurs upon the contact and clustering of ephrinBs in response to the binding and clustering of Eph receptors, and an Src family kinase is activated and phosphorylates the intracellular domain of B-type ephrins (Bruckner et al., 1997; Holland et al., 1996). This phosphorylation can result in the recruitment of a phosphatase that can regulate the signal via dephosphorylation (see Figure 22.1; Palmer et al., 2002).

There are phosphorylation-dependent and -independent associated signaling molecules and pathways for both receptors and ligands. One such protein, which binds B-type ephrins in a phosphorylation-independent manner, is the cytoplasmic protein PDZ-RGS3 (see Figure 22.1). This protein binds B ephrins through a PDZ domain, and it has a regulator of heterotrimeric G protein signaling domain. PDZ-RGS3 can mediate signaling from the ephrinB cytoplasmic tail. SDF-1, which is a chemokine with a G-protein–coupled receptor, acts as a chemoattractant for cerebellar granule cells, and this action is selectively inhibited by the engagement of a soluble EphB receptor

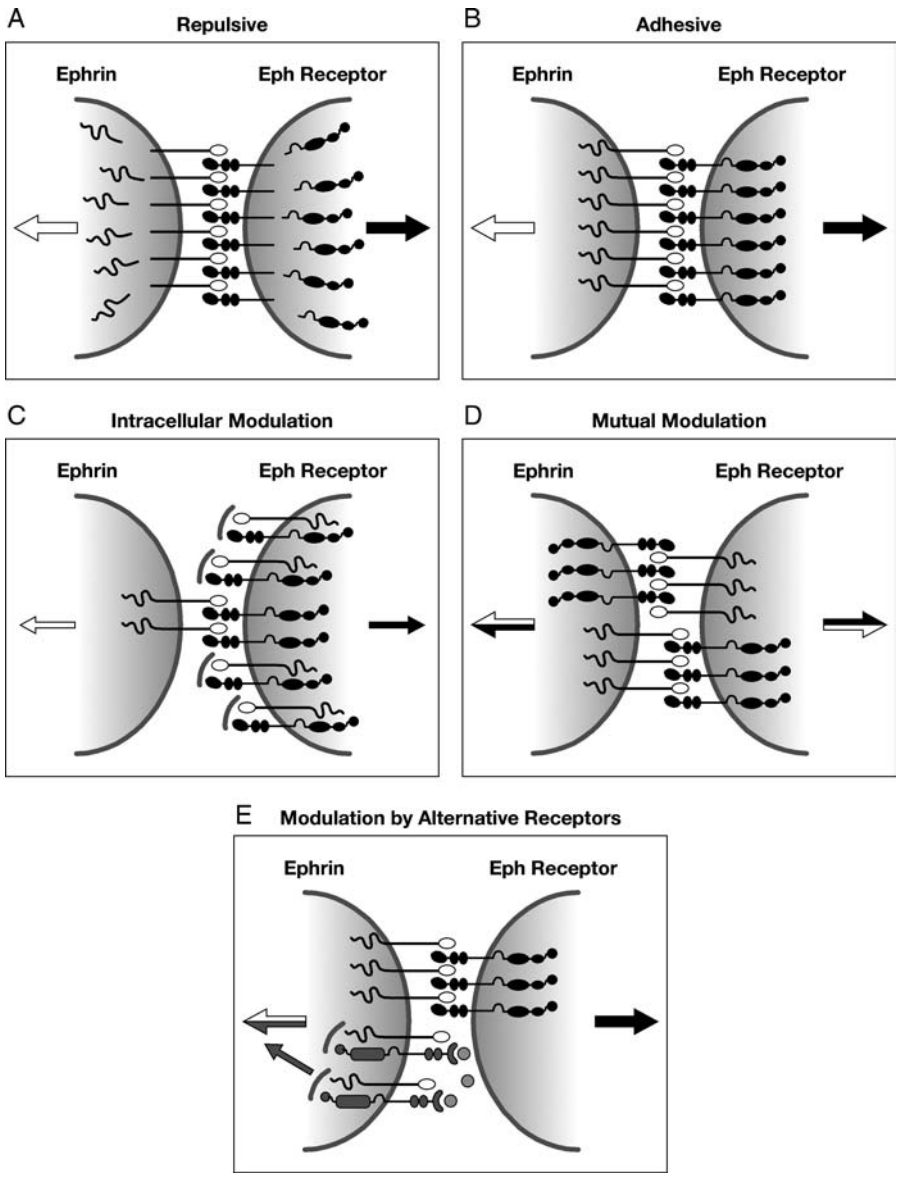

FIGURE 22.2 **Depiction of the adhesive and repulsive interactions resulting from Eph–ephrin interactions. A**, The mutual endocytic activation of both receptor and ligand initiated by higher-order clustering upon cell–cell contact and resulting in repulsive cues. *Open arrows* represent ephrinB signals, and *solid black arrows* represent EphB receptor signals. **B**, Eph–ephrin interactions can also result in the maintenance of cell surface molecules and adhesion. **C**, Interactions between ephrins and Eph receptors within the same cells may exist; only limited knowledge about this signaling exists, but repulsive cues are probably not elicited. *Smaller open arrow* represents reduced ephrin signals, and the *smaller solid black arrow* represents reduced or modified Eph signals. **D**, Intercellular signaling between Eph receptors and ephrins expressed within both cells may also exist and modulate signaling from both molecules. *Open/solid mixed arrows* represent modified or integrated signals from the reciprocal signals from ephrins and Eph receptors. **E**, Alternative growth factors and receptors may modulate signaling from ephrins (and possibly from Eph receptors). *Gray arrow* denotes alternative growth factor receptor signals, and *gray/open mixed arrow* represents modified or integrated signals from ephrins and the alternative growth factor receptors.

(Lu et al., 2001). Another protein, Dishevelled, which is a scaffold protein that is critical for the Wnt signaling pathway, has also been shown to bind B-type ephrins and to mediate the signals that affect cell sorting and movement via the Rho small GTPase pathway (Tanaka et al., 2003; Lee et al., 2006).

A phosphorylation-dependent association of ephrinB with Grb4, which is an adaptor protein, has been found, and this results in increased focal adhesion kinase (FAK) catalytic activity, the redistribution of paxillin, the loss of focal adhesions, and the disassembly of F-actin–containing stress fibers in cell culture (see Figure 22.1). Grb4 can also associate with other proteins implicated in cytoskeletal regulation, including Cbl-associated protein, the Abl-interacting protein-1, dynamin, P21 activating kinase1, heterogeneous nuclear ribonucleoprotein K, and Axin (Cowan and Henkemeyer, 2001).

Eph receptors can associate with proteins involved in regulating the Rho family of GTPases, which are intimately involved in regulating cell morphology, cell adhesion, and cell migration. One such protein is Ephexin, a guanine nucleotide exchange factor (RhoGEF) that activates RhoA and to some extent Cdc42 (see Figure 22.1; Shamah et al., 2001). Ephexin binds to the kinase domain of EphA4 through its Dbl homology and pleckstrin homology domains in the absence of receptor activation (Shamah et al., 2001). Receptor activation in response to ephrinA binding leads to increased Ephexin activity and the activation of RhoA. Another family member, Vms-RhoGEF, binds similarly but specifically on vascular smooth muscle cells (Ogita et al., 2003). Additionally, Vav2, which is a RhoGEF family member, has been shown to be recruited to the intracellular domain of Eph receptors and become transiently activated. Vav proteins are required for ephrin–Eph endocytosis and ephrin-induced growth cone collapse, thereby providing a molecular link between activated Eph receptors and Rac-dependent endocytosis (Cowan et al., 2005). A link to RhoA activation has been found for EphA3 through its binding of the adaptor protein CrkII (see Figure 22.1). CrkII binds to EphA3 upon receptor activation in response to ligand binding, and, through an unknown mechanism, this leads to RhoA activation (Lawrenson et al., 2002; Smith et al., 2004).

EphB receptors have a different subset of RhoGEFs, such as Intersectin and Kalirin, which are exchange factors for Cdc42 and Rac1, respectively (see Figure 22.1; Irie and Yamaguchi, 2002; Penzes et al., 2003). Intersectin, which is similar to Ephexin, binds to EphB2 in the absence of receptor activation, but it is not dependent on its Dbl homology and pleckstrin homology domains (Irie and Yamaguchi, 2002). Upon ephrinB binding, EphB2 is activated and recruits Kalirin to membrane clusters; however, it is unclear whether any direct or even indirect association between receptor and exchange factors exists (Penzes et al., 2003). Another protein with links to Rho signaling, Dishevelled, which also binds ephrinB1 (see Figure 22.1; Tanaka et al., 2003; Lee et al., 2006), has been found to bind activated EphB1 and EphB2, possibly through the adaptor protein Grb4 (Tanaka et al., 2003). Dishevelled associates with a Formin homology domain protein, Dishevelled-associated activator of morphogenesis (Daam1), and this interaction can lead to RhoA activation, presumably through a RhoGEF (Habas et al., 2001). Finally, a negative regulator of the small GTPase Ras, p120RasGAP, has been shown to bind phosphorylated EphB2 (Holland et al., 1997), and this protein can also associate with p190 RhoGAP, a negative regulator of RhoA activity (see Figure 22.1). Thus, there are several possible links between Eph receptors and ephrins and the regulators of cytoskeletal architecture.

II. OOCYTE MATURATION

In most animal species, oocytes arrest during meiotic prophase and complete meiosis in response to intercellular signaling during meiotic maturation. This process must be coordinated with other cellular aspects of oogenesis, including growth control, meiotic chromosome reorganization, and ovulation (Masui, 2001). The timing of the meiotic divisions with respect to fertilization varies among species, but there is conservation in the molecular signaling events of oocyte meiotic maturation among different animals. Studies of amphibian oocyte meiotic maturation led to the discovery of the maturation promoting factor (Masui, 2001; Tunquist and Maller, 2003), a complex of the Cdk1 catalytic subunit and the cyclin B regulatory subunit (Tunquist and Maller, 2003). Inhibitory phosphorylations of the complex are removed by the conserved Cdc25C phosphatase after stimulation with progesterone, which leads to nuclear envelope breakdown. M-phase exit and anaphase chromosome segregation require the function of a multi-subunit E3 ubiquitin ligase called the *anaphase-promoting complex/cyclosome* (Peters, 2005). In *C. elegans,* fertilization is required for completing meiotic divisions, whereas, in most vertebrates, fertilization occurs during metaphase II. A common requirement in this process, regardless of the animal system, is the dependence on signaling events initiated by fertilization that promote meiotic chromosome segregation and that initiate the embryonic program (Yamamoto et al., 2006).

In *C. elegans*, sperm use the major sperm protein (MSP) as a hormone to trigger oocyte meiotic maturation and ovulation. VAB-1 is a *C. elegans* Eph receptor has been identified as a receptor for MSP (Miller et al., 2003). VAB-1 inhibits oocyte maturation and the associated mitogen-activated protein kinase (MAPK) activation, and MSP suppresses VAB-1 function by competing with ephrinB2 (one of the four ephrins in *C. elegans*) for receptor binding. Consistent with the observed inhibition of MAPK in this system, EphB2 has been shown to inhibit MAPK in certain neuronal cells (Elowe et al., 2001; Tong et al., 2003). The data are consistent with the model that signaling through the VAB-1 Eph receptor and a POU homeodomain protein (CEH-18)–dependent pathway negatively regulates oocyte maturation and MAPK activation in hermaphrodite and female gonads. Sperm release MSP, thereby disrupting the pathway by binding to VAB-1 and an unidentified receptor. Binding antagonizes both VAB-1– and CEH-18–dependent signaling, which leads to the resumption of meiosis, the activation of MAPK, and ovulation. In the absence of CEH-18, VAB-1–mediated inhibition is dose dependent, which suggests that variations in MSP-mediated inhibition of VAB-1 signaling may be sufficient to induce a response. Eliminating VAB-1 and CEH-18 function removes the dependence of meiotic maturation and ovulation on the presence of sperm. Therefore, this meiotic control mechanism may represent a sperm-sensing control mechanism (Miller et al., 2003).

III. EARLY MORPHOGENESIS

The notochord consists of a rod-shaped body situated on the ventral aspect of the neural tube, and it constitutes the foundation of the axial skeleton. It is a mesoderm-derived structure, and it represents one of the earliest embryonic

structures to form. It lies at first between the neural tube and the endoderm, and it is then separated from these tissues by the mesoderm. From the mesoderm surrounding the neural tube and notochord, the skull and vertebral column are developed. Early dominant-negative and overexpression studies suggested that Eph–ephrin signaling may be involved in gastrulation movements in Xenopus embryos (Winning et al., 1996; Jones et al., 1998; Holder and Klein, 1999). In zebrafish, the expression of the ectodomain of the ephrinB ligand that can bind (but not activate) the Eph receptors caused defects in the morphology of the notochord and the prechordal plate by the end of gastrulation (Chan et al., 2001). An insertional disruption of the EphA2 gene in mice resulted in clear evidence of cell migration and positioning defects in the notochord (Naruse-Nakajima et al., 2001). EphA2-deficient notochordal cells spread into the tail bud, where normally the ligand ephrinA1 is expressed, and the newly derived notochord cells are excluded. This was the first genetic evidence that EphA2 and its ligands are involved in the positioning of the tail notochord through repulsive signals between cells expressing these molecules on the surface.

A. Segmentation

Segmentation serves to subdivide tissues into a series of repeating building blocks along either the body axis or the axial structures, thereby allowing for the elaboration of these uniform basic units into distinct structures during development. Eph–ephrin signaling has a recognized role in the segmentation of the hindbrain and the somites.

In vertebrates, paraxial mesoderm gives rise to trunk and limb skeletal muscles, the trunk skeleton, and regions of the trunk dermis and vasculature. A defining characteristic of all vertebrates is the metameric segmentation of musculoskeletal and peripheral nervous systems. This body plan arises from the primary segmentation of the paraxial mesoderm into cell blocks (somitomeres) that then give rise to tissue blocks called *somites,* and these eventually become the structures mentioned above. To appropriately create the boundaries needed for tissue separation and for the morphogenesis of such structures, the intermingling of distinct cell populations must be prevented, and thus requires control of cell motility. It has been demonstrated that Eph–ephrin signaling can block cell mixing. For example, embryonic zebrafish cells intermingle freely; however, if two cell populations are juxtaposed with one ectopically expressing EphA4 or EphB2 and the other expressing ephrinB2, then mixing is abolished in response to bidirectional signaling (Mellitzer et al., 1999). The ability to inhibit cell adhesion as well as cell motility makes Eph–ephrin signaling a formidable system for regulating tissue separation.

B. Hindbrain Segmentation

During development, the vertebrate hindbrain is transiently segmented into seven rhombomeres (Figure 22.3; see Chapter 9). Within the neuroepithelium, visible boundaries appear, cell contacts loosen, and eventually, boundary cells become discernible. Rhombomere boundaries restrict cell mixing, and a boundary forms usually between cells from odd- and even-numbered rhombomeres. Several Eph–ephrin family members are expressed in overlapping patterns that change during boundary formation (Sela-Donenfeld and Wilkinson,

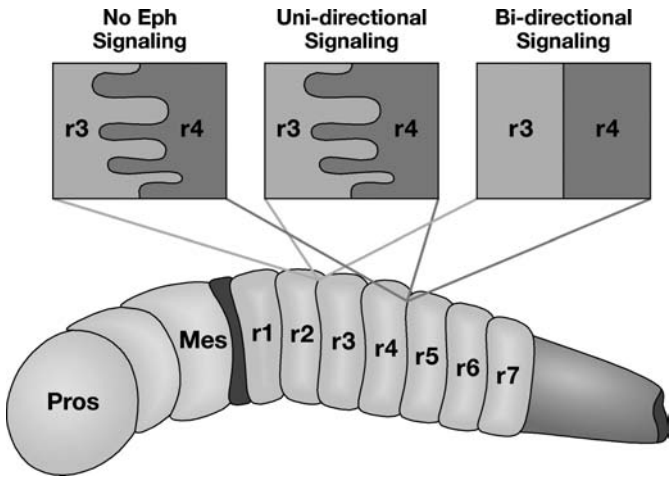

FIGURE 22.3 Boundary formation in the vertebrate hind brain in response to Eph and ephrin bidirectional signaling. Cartoon depiction of the developing vertebrate hind brain; boundaries are maintained only when bidirectional signaling from the Eph-receptor–expressing cells (r3) contact ephrin-expressing cells (r4). *r,* Rhombomere; *Pros,* prosencephalon (fore brain); *Mes,* mesencephalon (mid brain).

2005). In the mouse, Eph receptors and ephrins are generally expressed in a complementary manner, with Eph receptors in rhombomeres 3 and 5 and ephrins in rhombomeres 1, 2, 4, and 6. Although this is generally the case, there are exceptions in both mouse and Xenopus; for example, EphA2 is found in rhombomere 4, and it is coexpressed with the ephrinA3 ligand. In addition, EphB2 and EphB3 are transiently expressed in rhombomere 2 along with ephrinB1, ephrinB2, and ephrinB3 (Tepass et al., 2002).

In the zebrafish, the interaction of EphA4- and ephrinB2-expressing cells at rhombomere boundaries 3/4 and 4/5 demonstrates a clear example of Eph–ephrin involvement in segmentation (Tepass et al., 2002). The mosaic activation of EphA4 and EphB receptors by ephrinB2 leads to changes in the identity or movement of cells within r3/r5. Cells expressing ephrinB2 were restricted to the boundaries of r3/r5, whereas r2/r4/r6 cells are scattered throughout the segment (Xu et al., 1999). In zebrafish, valentino, which is a transcription factor that is necessary for the segmentation of the posterior hindbrain, is required for the mutually exclusive expression of EphB4 and ephrinB2 in the caudal hindbrain. With the use of mosaic cells from zebrafish embryos deficient in valentino, it was shown that spatially inappropriate Eph signaling underlies the repulsion of val^- cells from r5/6 (Cooke et al., 2001). These and other findings suggest that the localized activation of Eph receptors and ephrin ligands (bidirectional signaling) at complementary interfaces causes a mutual repulsion that precludes cell mixing (see Figure 22.2; Sela-Donenfeld and Wilkinson, 2005). Of course, this bidirectional signaling may be responsible for more than just repulsion at the interface. Mosaic experiments in which embryonic cells have EphA4 expression blocked by antisense morpholinos show that, when they are transplanted onto a wild-type background, the $EphA4^-$ cells segregate to the periphery of r3/r5 borders, whereas the $EphA4^+$ cells maintain the center (Cooke et al., 2005). These experiments suggest an adhesive role within the segments for Ephrin/EphA4 while

demonstrating a repulsive role at the segment interfaces. There are still many unresolved questions: Does Eph receptor activation mediate adhesion and repulsion? Does the response depend on the overlap between Eph-receptor and ephrin expression and whether a specific ephrin will induce adhesion versus repulsion (Poliakov et al., 2004)? Does the level of Eph receptor clustering determine whether adhesion or repulsion occurs (Sela-Donenfeld and Wilkinson, 2005)? Do other alternative growth factors and molecules modulate adhesion versus repulsion at the hindbrain segment boundaries?

IV. CELL MIGRATION AND POSITIONING

The Eph–ephrin system plays a role in cell migration and ultimately cell positioning in an array of morphogenetic events during development, including neural crest migration, cerebellar granule cell migration, retinal progenitor cell movement, epithelial cell migration, and axon guidance.

A. Neural Crest

Neurulation is the process during which the central nervous system is formed as the neural plate bends and folds into the neural tube. Presumptive neural crest cells are localized at the border of the neural plate and the nonneural ectoderm. During this morphogenetic process, neural crest precursors are found within the neural folds and later localize to the dorsal portion of the completely closed neural tube. These cells subsequently undergo an epithelial to mesenchymal transition, delaminate from the neuroepithelium, and migrate from the neural tube to various embryonic sites. These multipotent cells will differentiate into a diverse array of cell types, including cartilage and bones, neurons and glia of the peripheral nervous system, melanocytes of the skin, and smooth muscle of the heart (see Chapter 26).

Despite the vast movement and diverse structures in which neural crest derivatives contribute, the basis for neural crest formation can be found in segmental patterning. For example, craniofacial development is intrinsically related to segmental patterning of the neural tube that results in the generation of neuromeres. In vertebrate embryos, segmentally arranged cranial structures, such as the branchial arches and sensory ganglia, are formed on the basis of this metamerism. The segmental organization of the hindbrain (described previously) is particularly important for the patterning of the neural crest cell migration pathways. The Eph–ephrin system has been shown to contribute to the migration of trunk and branchial arch neural crest cells.

In chick and murine embryos, trunk neural crest cells migrate through the anterior rather than the posterior half of each somite (Barembaum and Bronner-Fraser, 2005), and this segmental migration underlies the formation of the repeated pattern of dorsal root and sympathetic ganglia (Kalcheim, 2000). Somites guide neural crest cells and motor axons, presumably through the combination of attractive cues within the anterior region of each somite and repulsive cues within the posterior portion. EphrinB proteins have been shown to repel trunk neural crest cells and motor axons that express cognate EphB receptors *in vitro* (Krull et al., 1997; Wang and Anderson, 1997). EphrinBs are expressed in the posterior portion of somites, and *in vivo* experiments in chick trunk explants show that EphB–ephrinB interactions

are required to prevent neural crest cells from entering the posterior portion of somites (Krull et al., 1997).

The segmental migration of neural crest also occurs from rhombomeres to specific branchial arches, where they differentiate to form specific craniofacial structures (Barembaum and Bronner-Fraser, 2005). In Xenopus embryos, premigratory branchial neural crest is segmented into adjacent groups of cells that are destined to enter the four branchial arches. Complementary expression of ephrinB2 and of EphA4 or EphB1 in the adjacent migratory streams of neural crest cells has been demonstrated, and the disruption of the EphA4 receptor leads to the cell-autonomous mistargeted migration of these cells into adjacent territories (Smith et al., 1997). In a complementary experiment in the mouse, a knockout of ephrinB2 appeared to block the proper migration of neural crest cells into the second branchial arch (Adams et al., 2001). EphrinB1 was shown to be required for branchial neural crest cell migration, and mice deficient in ephrinB1 had a cleft palate phenotype that was indicative of a neural crest defect (Davy et al., 2004). The complete ablation of ephrinB1 resulted in defects in several neural-crest–derived tissues, incomplete body wall closure, and abnormal skeletal patterning. The conditional deletion of ephrinB1 demonstrated that ephrinB1 acts autonomously in neural crest cells and that it controls their migration (Davy et al., 2004). Of particular interest is the fact that most previous studies demonstrated that ephrins act as repellent cues for Eph-receptor–expressing neural crest cells. In contrast, by creating a mutation in the PDZ binding motif within the intracellular domain of ephrinB1, it was shown that reverse signaling through the intracellular domain of this ligand is required for proper neural crest cell migration (Davy et al., 2004). Thus, ephrinB1 acts as both a ligand and a receptor in neural crest during embryogenesis.

Neural crest cells that are destined to become neurons and glia only migrate ventrally, and they are precluded from migrating dorsolaterally into the skin. However, neural crest cells specified to be melanoblasts migrate along a dorsolateral pathway. In chick, the disruption of Eph–ephrin interactions by the addition of soluble ephrinB ligand to trunk explants led to the inappropriate migration of early neural crest cells into the dorsolateral pathway (Santiago and Erickson, 2002). Moreover, when Eph receptor signaling is disrupted *in vivo*, a subpopulation of melanoblasts is prevented from migrating dorsolaterally, thus suggesting that ephrinB ligands promote the dorsolateral migration of melanoblasts (Santiago and Erickson, 2002). Transmembrane ephrins may act as bifunctional guidance cues when they initially repel early migratory neural crest cells from the dorsolateral path where ephrinBs reside, and they may then later stimulate the migration of melanoblasts into this pathway. The mechanism by which ephrin signals repulsion and/or attraction in the dorsolateral pathway remains a fertile area for exploration.

B. Epithelial Migration and Positioning

Studies in *C. elegans* were the first to show that Eph–ephrin interactions played a pivotal role in epithelial migration. One Eph receptor (VAB-1) and four ephrins (1, 2, 3, and 4) exist in *C. elegans*. Null mutants in VAB-1 display a defect in epidermal cell movement during ventral enclosure (George et al., 1998; Chin-Sang et al., 1999). Single ephrin1–3 mutants show mild epidermal

phenotypes, and triple mutants phenocopy the VAB-1 null defect. Interestingly, a mutation in ephrin4 displays synergistic interactions with mutations in the VAB-1 receptor and in ephrin1, which indicates that this ligand may function independently of the VAB-1 Eph receptor in morphogenesis (Chin-Sang et al., 2002). Moreover, a recent study suggests that the VAB-1 Eph receptor has a genetic interaction with another neuronal receptor, SAX-3/Robo, for proper embryogenesis. An analysis of SAX-3 mutants showed that SAX-3/Robo functions with VAB-1 during gastrulation for cleft closure and ventral epidermal enclosure (Ghenea et al., 2005).

Eph–ephrin interactions also have been shown to be important players in the precise positioning of intestinal epithelial stem cells within the lumen of the mammalian intestine. The proliferative compartment of the small intestinal epithelium is composed of millions of small invaginations known as crypts, which contribute to the renewal of the intestinal epithelium during adulthood. A small number of intestinal stem cells at the bottom of each crypt give rise to a population of progenitor cells that migrate toward the intestinal lumen. During their migration, progenitors become committed toward a particular lineage (goblet, absorptive, or enteroendocrine), and they migrate to the tip of the villus, whereas Paneth cells in the small intestine migrate toward the bottom of the crypts.

The Wnt signaling pathway plays a key role in the intestinal epithelium by stabilizing β-catenin via inhibition of an adenomatous polyposis coli (APC)-axin-glycogen synthase kinase (GSK)-3 complex responsible for targeting β-catenin for destruction. Stabilized β-catenin accumulates and localizes to the nucleus, where it interacts with T cell factor (TCF) transcription factors that activate Wnt target genes (Bienz and Clevers, 2000).

In the small intestine of newborn mice, EphB2 and EphB3 show overlapping expression in the highly proliferative intervillus pockets of the epithelium. Only ephrinB1 appears to be highly expressed by all epithelial cells, with the exclusion of those localized at the bottom of intervillus pockets. Thus, ephrinB1 and the cognate EphB2 and EphB3 receptors are expressed in complementary domains within the intestinal epithelium, and Wnt signaling can upregulate EphB2 and EphB3 receptors while downregulating ephrinB1 (van de Wetering et al., 2002). The disruption of EphB2 and EphB3 genes in mice leads to a disruption of the restriction of cell intermingling and the normal allocation of cell populations within the intestinal epithelium. In EphB2/EphB3 null newborn mice, the proliferative and differentiated populations inappropriately intermingle, which suggests that the presence of the EphB receptors in wild-type proliferative regions actively restricts the migration of ephrinB1-expressing differentiated cells (Batlle et al., 2002).

In adult EphB3 null mice, the quiescent differentiated Paneth cells are defective in their ability to localize to the bottom of the crypt, and they instead scatter along the crypt and the villus; this indicates that EphB3 is required in these cells for appropriate migration and positioning. Thus, β-catenin and TCF couple proliferation and differentiation with the sorting of cell populations through the EphB–ephrinB system (Batlle et al., 2002).

C. Cerebellar Granule Cell Migration

Further evidence supporting a role for ephrinB in cell migration comes from studies showing that reverse signaling (through the ephrinB intracellular

domain) can impinge on the attractive guidance of the SDF-1/CXCR4 system (Lu et al., 2001). In the early postnatal cerebellum, granule cell precursors actively proliferate in the superficial external granular layer and then radially migrate across the molecular layer, enter the Purkinje cell layer, and complete their migration into the deep stratum of the inner granule layer (Komuro and Yacubova, 2003). In the prenatal and early postnatal cerebellum, stromal-cell–derived factor 1 (SDF-1) expression is restricted to the superficially located pial membrane, whereas granule cell precursors express its receptor, CXCR4. SDF-1 induces chemotactic responses in granule cell precursors, and, along with CXCR4, it plays a crucial role in retaining granule cell precursors in the external granular layer. In particular, SDF-1 prevents inward migration of CXCR4-positive granule cells by attracting them toward the pial membrane (Komuro and Yacubova, 2003).

Although the expression of SDF-1 or CXCR4 is sustained in the early postnatal cerebellum, postmitotic granule cells divert from this chemoattractive system and migrate inward toward the inner granule layer. A molecular mechanism that may explain this process involves ephrinB2 and EphB2, which are expressed in the external granular layer before the inward migration of postmitotic granule cells (Lu et al., 2001). It was observed that the chemoattraction of granule cells to SDF-1 is inhibited by a soluble EphB2 receptor, the mode of action of which involves reverse signaling through ephrinB2. The reverse signaling is mediated by PDZ-RGS3, a cytoplasmic protein that constitutively binds ephrinB2 in the C-terminus and that inhibits heterotrimeric G-protein signaling through its GTPase activating protein (GTP hydrolysis) function (Lu et al., 2001). These results suggest that the inward migration of granule cells is the result of the loss of responsiveness to SDF-1 and that this may be mediated, at least in part, by the EphB2/ephrinB2 signaling.

D. Eye Field Morphogenesis

As the central nervous system is regionalized, a subset of the anterior neural plate is specified as the eye field. Potential retinal progenitors need to be positioned within the eye field to receive the local environmental signals that will direct their ultimate fates (Saha et al., 1992; Li et al., 1997). Only after these steps are accomplished do the steps of eye organogenesis, cellular lamination, and phenotype specification occur. There are three major morphogenetic movements that bring embryonic cells into the correct position to form the retina. During gastrulation, epiboly movements position the dorsal ectoderm in register with signaling centers that induce anterior neural ectoderm. During the formation of the neural plate, forebrain, and eye field, cells disperse and intermix with adjacent lineages (Bauer et al., 1994). Initially, a single eye field that extends across the midline of the early neural plate is formed, but cells are later displaced or move laterally to form the left and right eye primordia (Varga et al., 1999), which are separated by medially located diencephalic precursors.

An accepted hypothesis of how the eye field forms is that signals from surrounding anterior structures regionalize the anterior neural plate (Saha et al., 1992; Perron and Harris, 2000). The presumptive eye field then expresses several transcription factors that initiate the retina developmental program. However, there also is evidence that cellular movements during gastrulation and neurulation are critical (Chuang and Raymond, 2001; Kenyon et al.,

2001). In Xenopus, with the use of antisense morpholinos to ephrinB1, it was shown that ephrin signaling during gastrulation is required for retinal progenitors to move into the eye field and that this signaling can be modified by activating the fibroblast growth factor (FGF) pathway. FGF receptor2 and FGF receptor4 are expressed in the anterior ectoderm at neural plate stages (Golub et al., 2000) in close proximity to the domain of ephrinB1 expression in the anterior neural plate, which itself overlaps with the eye field (Jones et al., 1997). A receptor for ephrinB1 (EphB2) is also expressed during the gastrulation and neural plate stages in these domains (Tanaka et al., 1998). Thus, components of both the FGF and ephrin–Eph signaling pathways are expressed in coincident temporal and spatial patterns that would allow them to interact and regulate retinal progenitor movements. Furthermore, the FGF receptor can complex with and induce the phosphorylation of ephrinB1, which results in effects on cell adhesion when these are ectopically expressed in Xenopus (see Figures 22.1 and 22.2; Chong et al., 2000). Using gain- and loss-of-function analyses, it was shown that FGF receptor activity inhibits cell dispersal in the developing Xenopus neural plate and that decreased dispersal correlates with a decrease in the acquisition of retinal fates (Moore et al., 2004). EphrinB1 activity is found to have the opposite effect, promoting cell dispersal and thus favoring the acquisition of retinal fates. A mutant of ephrinB1 was unable to induce retinal progenitor movement into the eye field, thus indicating that signaling through the intracellular domain of ephrinB1 was critical for appropriate movement (Moore et al., 2004). Of particular interest was the indication that signaling from the FGF receptor can regulate ephrin signaling and play a critical role in establishing the bona fide retinal progenitors in the anterior neural plate.

Recent evidence indicates that ephrinB1 signals via its intracellular domain to control retinal progenitor movement into the eye field by interacting with Xenopus Dishevelled (Xdsh) and by co-opting the planar cell polarity (PCP) pathway (see Figure 22.1; Lee et al., 2006). Blocking Xdsh translation using antisense morpholino oligonucleotides prevented retinal progeny from entering the eye field, similar to the morpholino-mediated loss of ephrinB1 (Moore et al., 2004). The overexpression of Xdsh can rescue the phenotype induced by a loss of ephrinB1, and this rescue, as well as a physical association between Xdsh and ephrinB1, is completely dependent on the C-terminal Dishevelled/Egl10/Pleckstrin domain of Xdsh. This domain is critical for Dishevelled to mediate the noncanonical Wnt/PCP signaling known to affect gastrulation movements (Wallingford and Habas, 2005). Similar gain- and loss-of-function experiments suggest that Dishevelled mediates ephrinB1 signaling via downstream members of the PCP pathway during eye field formation (Lee et al., 2006).

E. Retinotectal Axon Positioning

In addition to playing a role in the formation and positioning of progenitors in the retinal field, the Eph–ephrin system plays a key role in axon guidance in the retina. In the visual system, retinal ganglion cells project axons to establish a connection to the target tissue in the midbrain (tectum in chick and superior colliculus [SC] in mouse). Maintaining the appropriate spatial organization of these connections along the anterior–posterior (AP) and dorsal–ventral (DV) axes allows for the accurate transmission of visual images to

the brain. The graded expressions of Eph receptors and ephrins are critical regulators of this process. For example, along the AP axis, axons expressing high levels of EphA receptors project from the posterior retina to the anterior SC, which presents low levels of ephrinA2 and ephrinA5. Conversely, axons displaying low levels of EphA receptors in the anterior retina project to the posterior SC, which presents high levels of ephrinAs (Poliakov et al., 2004). Both *in vitro* and *in vivo* assays show that axons displaying high levels of EphA receptors are more sensitive than low-level expressors to repulsion by regions displaying relatively low levels of ephrinAs (Wilkinson, 2001). A gradation of ephrinA5 levels in retinal axons can modulate responses to tissues displaying ephrinA ligands (Hornberger et al., 1999). Thus, a model for axon guidance emerges where retinal axons project through a gradient of ephrinA until repulsion occurs, and this is determined by the gradient of responsiveness along the AP axis of the SC. However, genetic studies in mouse show that the loss of ephrinA2 and ephrinA5 in the SC still allows for axons to project to the SC, but the topographic organization is disrupted (Feldheim et al., 2000). These and other studies led to the suggestion that retinal axons compete with each other for projection to the SC, such that axons possessing low levels of EphA receptors will have the advantage over those with high EphA levels for projecting to regions with high ephrinA levels.

Recent evidence indicates that ephrinAs may switch between acting as attractants or repellants, depending on the level of EphA activation (Hansen et al., 2004). An axon growth assay was developed to systematically vary both retinal position and ephrin concentration. EphrinA2 inhibited growth at high concentrations, but it promoted growth at lower concentrations. Moreover, the concentration at which promotion transitions to inhibition varied topographically with retinal position and level of EphA receptor levels (Hansen et al., 2004). A similar mechanism may apply to the DV axis of the retinal topographic system. In the DV axis, EphB receptors are expressed in a low-to-high gradient, whereas ephrinBs are expressed in a low-to-high gradient in the lateral-to-medial axis in the SC. Unlike the EphA–ephrinA system in the AP axis, ventral axons display high levels of EphB receptors that project to the dorsal SC, which presents high ephrinB levels. Conversely, dorsal axons displaying high levels of ephrinBs project to the ventral SC, which presents high levels of EphBs (Palmer and Klein, 2003) and which suggests an attractive response to the interaction. Genetic evidence provided by EphB2/EphB3 null or kinase-defective mice shows that a lack of these receptors will shift the ventral axon termination zones laterally in the SC, where lower levels of ephrinB1 reside (Hindges et al., 2002). EphrinB1 selectively controls the directional extension of retinal axon side branches at a particular DV position through either attraction or repulsion. Axons terminating ventrally will extend branches dorsally up the gradient of ephrinB1 (attractive), whereas axons terminating dorsally will extend branches ventrally, repelled down the ephrinB1 gradient (McLaughlin et al., 2003).

An additional level of precision regarding branch positioning comes from cross-talk with the Wnt signaling pathway, where Wnt3A is found in a similar expression gradient as ephrinB1, and the alternative Wnt receptor known as Ryk is expressed in a similar gradient as EphBs (Schmitt et al., 2006). When Wnt is over-expressed in the tectum, the RGC axons are repelled from the Wnt expression zone. Moreover, when a dominant-negative Ryk is expressed in RGC axons, a medial shift occurs in their termination zone in the tectum.

The opposite result is observed for RGC axons that have mutant EphB. These data strongly suggest a model where the Wnt3A concentration gradient provides a laterally directing repellent force to counterbalance the medially directing attractive force from EphrinB on RGC axon branches (Schmitt et al., 2006).

F. Dendritic Spine Morphogenesis

An example of Eph–ephrin interactions that play a role in cellular morphologic change is observed in dendrites. The morphogenesis of dendritic spines, which are the major sites of excitatory synaptic transmission in the brain, is important in synaptic development and plasticity. Dendritic spines are small protruding structures that form on the neuronal dendrite surface. These filopodial protrusions establish synaptic connections with axons, and they receive excitatory synaptic input. These dendritic filopodia undergo dramatic changes in morphology as they mature, yielding mushroom-shaped, spine-like projections. These dynamic morphologic alterations are controlled by the actin cytoskeleton, and they are coincident with postsynaptic specialization (Murai and Pasquale, 2005). EphA4, EphA7, EphB2, and EphB3 are localized in the dendritic spines of hippocampal neurons. In addition, Eph receptors bind various PDZ domain proteins in the postsynaptic site, such as PICK1, GRIP, syntenin, and AF-6 (Irie and Yamaguchi, 2004). Thus, Eph receptors are involved in synapse development. The activation of EphB receptors leads to a direct interaction with the N-methyl-D-aspartate–type glutamate receptors, whereas disrupting EphB kinase activity reduces the number of postsynaptic specializations in cultured cortical neurons (Irie and Yamaguchi, 2004).

Syndecans are transmembrane heparan–sulfate proteoglycan mediators of signaling involved in spine morphogenesis. Syndecan-2 is tyrosine phosphorylated in cultured hippocampal neurons and coimmunoprecipitates with EphB2 from cultured neurons as well as synaptosomes from mouse brain. These data suggest that EphB2 may regulate syndecan-2 at the postsynaptic region. A dominant-negative form of EphB2 blocked the ability of syndecan-2 to induce spine formation, and the treatment of hippocampal cultures with clustered ephrinB induces spine formation (Penzes et al., 2003). In genetic studies, EphB2 null mice do not display an overt phenotype in the dendritic spines; therefore, further investigations regarding functional redundancy with other Eph receptors and ligands will be required for further clarity.

Other mechanisms by which EphBs drive spine formation may involve the recruitment of PDZ proteins (Torres et al., 1998) and the activation of RhoGTPases through RhoGEFs such as Intersectin-1, Kalirin-7, and Tiam 1 (see Figure 22.1; Irie and Yamaguchi, 2002; Penzes et al., 2003; Tolias et al., 2005). Recently, it has also been shown that the ligand-induced activation of EphB2 in cultured hippocampal neurons results in the shortening of dendritic filopodia through the assembly of a protein complex that includes FAK, Src, Grb2, and paxillin. The disruption of FAK expression or RhoA activity also blocked EphB-mediated dendritic filopodia morphogenesis, which suggests that EphB receptors are upstream regulators of FAK in dendritic filopodia and that FAK-mediated RhoA activation contributes to the assembly of actin filaments in dendritic spines.

In addition to the effects of EphB receptors, it has been demonstrated that interaction between ephrinA3 on astrocytes and EphA4 on postsynaptic sites

controls the retraction of dendritic spines in the adult hippocampus (Murai et al., 2003). Moreover, when CA1 cells in hippocampal slice cultures were transfected with a kinase-inactive EphA4 receptor construct, abnormal and disorganized spines were observed that were similar to those seen in EphA4 null mice. Therefore, the endogenous ephrinA3 ligand may be prevented from properly regulating spine shape (Murai et al., 2003). One model suggested by this work is that spine shape is maintained by the interaction of ephrinA3 on the surface of astrocytes with the EphA4 receptors expressed in the plasma membrane of spines. The spine shape changes result in the low-level activation of EphA4, which initiates the retraction of the spine away from the astrocyte (Murai et al., 2003; Thompson, 2003). It is possible that retraction of the spine neck is also mediated through changes in actin polymerization.

G. Angiogenesis

The formation of the vasculature represents an excellent example of how the Eph–ephrin system can control the assembly of tubular structures through the regulation of their adhesive and repellent or migratory effects. During early embryogenesis, somites begin to form; when this occurs, a primitive vascular network is also established by the process of vasculogenesis. Mesoderm-derived hemangioblasts give rise to blood islands, and these cells proliferate and differentiate to form the precursors of the endothelial cells of the vessel wall, the angioblasts, and the precursors of the hematopoietic cells (Patan, 2000; see Chapter 33). Fusion of the blood islands results in the derivation of the primary vascular plexus inside the embryo, and the process of remodeling begins. During remodeling, the number and location of vascular segments are rearranged where vascular fusion reduces the number of segments and gives rise to larger vessels. In some locations, larger vessels are remodeled into a network of smaller ones, subsequently increasing the number of segments and leading to the formation of a secondary plexus that is expanded by the process of angiogenesis. During angiogenesis, preexisting blood vessels are expanded through endothelial sprouting and microvascular growth (Patan, 2000). Thus, the formation of the large embryonic vessels is accomplished through the morphogenetic events of vessel fusion and splitting that induce further growth and remodeling by the process of angiogenesis.

Although several Eph receptors and ephrins are expressed in endothelial cells (Poliakov et al., 2004), EphB2 and ephrinB2 are the most clearly implicated in the process of angiogenesis. The EphB4 receptor is expressed predominantly in veins, whereas ephrinB2 is restricted to the arteries (Adams, 2002). Genetic studies in mice that disrupt either ephrinB2 or EphB4 result in defective remodeling during angiogenesis (Adams, 2002). These studies led to the concept that the interactions of the Eph–ephrin system are involved in demarcating the arterial and venous identities of blood vessels via repulsive interactions between the ligand–receptor pair at the boundaries. Wider vascular defects were observed in the ephrinB2 null mice, including defects in the large vessel precursor as well as intersomitic vessels (Adams, 2002). The role of reverse or intracellular signaling from ephrinB2 in this process is more enigmatic. Adams et al. (2001) showed that, in a knock-in mouse in which the cytoplasmic domain of ephrinB2 was missing, angiogenic defects similar to the ephrinB2 null mouse were demonstrated.

By contrast, another study showed no angiogenic phenotypes when the cytoplasmic domain of ephrinB2 was replaced with β-galactosidase in mice, although cardiac defects led to postnatal mortality (Cowan et al., 2004). Clarification of the role of reverse signaling in angiogenesis may require the generation of specific mutations within the C-terminus of ephrinB2 in mice or a complementary experiment deleting the kinase domain of the EphB4 receptor. Thus, the role of reverse signaling through the intracellular domain of ephrinB2 in this process is still unclear.

In Xenopus, ephrinB ligands are expressed complementary to EphB4 in the somites adjacent to the migratory pathways taken by intersomitic veins during angiogenic growth (Figure 22.4, A; Helbling et al., 2000). The expression of dominant-negative EphB4 receptors or the misexpression of ephrinB ligands in Xenopus embryos disrupts the proper migration and formation of intersomitic blood vessels and vasculature. These findings demonstrate that EphB4 and B-class ephrins act as regulators of angiogenesis possibly by mediating repulsive guidance cues to migrating endothelial cells. Further evidence for ephrin expression in surrounding tissues contributing to proper vascularization comes from transgenic mice that express ephrinB2 under the control of a ubiquitous and constitutive promoter (Oike et al., 2002). These mice displayed an abnormal segmental arrangement of intersomitic vessels, whereas such anomalies were not observed in mice overexpressing ephrinB2 only in vascular endothelial cells. This finding suggests surrounding tissue that expresses ephrinB2 alters the migration of endothelial cells expressing EphB receptors in the intersomitic region. Collectively, all of these studies suggest that EphB4–ephrinB2 signaling between endothelial cells and surrounding mesenchymal cells plays an essential role in vasculogenesis, angiogenesis, and vessel maturation.

V. EPH/EPHRINS IN CANCER

A. Angiogenesis in Tumorigenesis

In two recent studies, EphB4 and ephrinB2 have been linked to angiogenesis in tumor formation. One study used a green-fluorescent-protein–tagged EphB4 lacking the kinase domain to differentiate between EphB4 and ephrinB2 signaling. Interestingly, the expression of the EphB4 kinase mutant in breast cancer cells increased tumor growth in a mouse xenograft model. Examination of the tumors revealed that ephrinB2 is primarily expressed in the vasculature and that the EphB4-kinase-mutant–expressing tumors displayed an increase in the size of blood vessels. In support of an effect on the vasculature, the extracellular domain of EphB4 attracted endothelial cells in vitro and stimulated endothelial cell invasion, survival, and proliferation. A model is proposed in which EphB4 on the tumor surface promotes the formation of blood vessels from ephrinB2-expressing vascular cells, which results in increased tumor growth (Noren et al., 2004). The second study showed that ephrinB2 and EphB4 are coexpressed by blood vessels of human and experimental malignant brain tumors (Erber et al., 2006). Endothelial overexpression of wild-type EphB did not affect normal vascular initiation of the tumors, but it had two fundamental effects on the subsequent organization of the vascular system. It markedly affected vascular morphogenesis (as

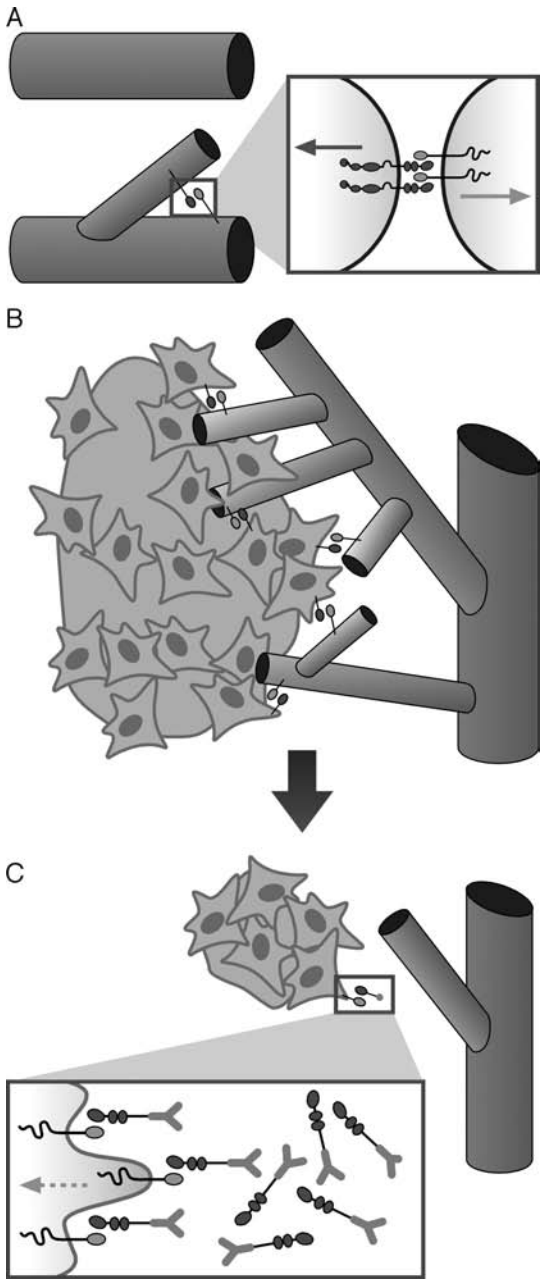

FIGURE 22.4 **Eph–ephrin interactions are important during normal angiogenesis, and they represent amenable therapeutic targets during tumor angiogenesis. A**, During normal angiogenesis *(red tubular structures)*, Eph–ephrin interactions induce branching and sprouting. Inset shows interaction between receptor and ligand during cell contact. **B,** During tumorigenesis *(green cells)*, Eph–ephrin interactions may play a role in sprouting and branching into tumor tissues. **C,** Using soluble Eph receptor ectodomains as competitive inhibitors of Eph–ephrin interactions may result in reduced tumor angiogenesis. Inset shows soluble Eph receptor interaction with ligands. (Adapted from Palmer and Klein, 2003. See color insert.)

evidenced by a switch from angiogenic sprouting to circumferential vessel growth), and it reduced the permeability of tumor blood vessels. Moreover, kinase-dead EphB4 induced the same vascular changes, thereby suggesting that reverse signaling via ephrinB2 represents the predominant signaling pathway in this context (Erber et al., 2006).

EphA2 and its ligand ephrinA1 appear to be expressed during angiogenesis in the adult, and these proteins have also been implicated in tumor angiogenesis (Ogawa et al., 2000). The complementary expression of ephrinA1 in tumor cells and the EphA2 receptor in tumor-associated blood vessel endothelium was reported (Brantley et al., 2002). Moreover, soluble EphA receptor extracellular domains inhibited tumor angiogenesis in cutaneous window assays and tumor progression *in vivo* (see Figure 22.4). Although the soluble receptor domains had no direct effect on tumor cell growth or apoptosis in culture, the migration of endothelial cells in response to tumor cells was blocked, which suggests that the soluble receptor inhibited blood vessel recruitment by the tumor (see Figure 22.4). In a later study, metastatic mammary adenocarcinoma cells transplanted into EphA2-deficient mice displayed decreased tumor volume, tumor cell survival, microvascular density, and lung metastasis relative to tumor-bearing littermate controls. Analysis of endothelial cells isolated from EphA2-deficient animals showed impaired survival; in addition, they failed to incorporate into tumor microvessels in vivo, and they displayed impaired tumor-mediated migration in vitro (Brantley-Sieders et al., 2005). These studies suggest that EphA2 receptor function is required in the tumor microenvironment for angiogenesis and metastatic progression.

B. Eph Expression in Tumors

In addition to a role for Eph and ephrin molecules in angiogenesis during tumor formation, these proteins have been implicated in playing a role in tumor invasion and progression. The cell–cell adhesion system mediated by cadherin plays a critical role in normal developmental morphogenetic processes and in metastasis. Both processes depend on the ability to form and disassemble cell–cell contacts, and the inactivation of this adhesion system has been shown to play a critical role in cancer invasion and metastasis. There is compelling evidence that Eph receptor tyrosine kinases and their ephrin ligands are either regulated by or control cell–cell adhesion complexes (Pasquale, 2005). Eph receptor tyrosine kinases and ephrins are frequently overexpressed in a variety of cancers and tumor cell lines, including breast, prostate, non–small-cell lung and colon cancers, melanomas, and neuroblastomas and particularly in metastatic tissue (Wimmer-Kleikamp and Lackmann, 2005).

Although the expression of Eph receptors and ephrins is generally extremely low in normal adult tissues, overexpression has been found to correlate with increased invasiveness and the aggression of several different tumors, including carcinomas of the breast, colon, and kidney, as well as melanoma, neuroblastomas, and ovarian and prostate cancers (Wimmer-Kleikamp and Lackmann, 2005). In malignant melanoma, it was found that the expression of EphA2 and EphB3 correlated with cancer progression and that it was consistent with a role in neural crest migration (Helbling et al., 2000; Krull et al., 1997). EphA3 and ephrinA1 are also highly expressed in advanced malignancies (Wimmer-Kleikamp and Lackmann, 2005).

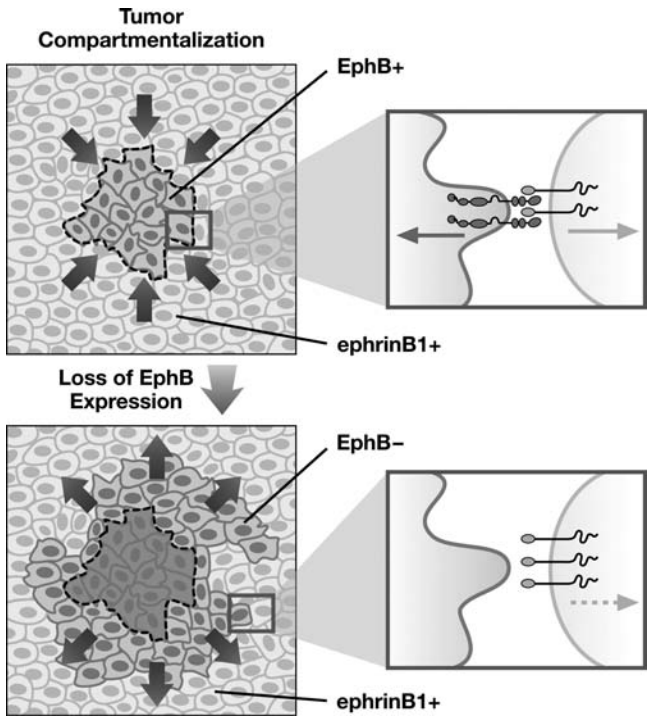

FIGURE 22.5 **Loss of Eph–ephrin interactions may result in tumor invasion.** Tumors cells *(green)* may be confined to small foci as a result of the bidirectional signaling *(top inset)* maintained by the expression of ephrins in surrounding normal tissue *(tan cells)*. During tumorigenesis, the loss of Eph expression in tumor cells leads to unidirectional signaling *(bottom inset)* and allows tumor cells to invade surrounding tissue. Adapted from Clevers and Batlle, 2006. (See color insert.)

Although some initial studies found EphB receptors to be overexpressed in colon cancers, recent evidence suggests that EphB2 and EphB3 may be tumor suppressors that are downregulated during the metastatic development of colon cancer (Clevers and Batlle, 2006). It was found that low- and medium-grade tumor areas were enriched in $EphB^{+}$ cells, whereas clusters of $EphB^{-}$ correlated with high-grade areas (Figure 22.5). Thus, the repression of EphB expression in a subset of tumor cells correlates with the acquisition of malignancy. In genetic studies in mice, tumor progression shows a robust acceleration in the large intestine of APCmin/+ mice also lacking EphB activity, which results in the development of aggressive colorectal adenocarcinomas (see Figure 22.5; Batlle et al., 2005). Two recent studies have shown that the extent of EphB2 repression in colorectal cancer correlates inversely with patient survival (Jubb et al., 2005; Lugli et al., 2005), and another determined frameshift mutations to be an important player in colorectal cancer progression (Alazzouzi et al., 2005).

In breast cancer, there are reports that show either a strong correlation (Berclaz et al., 2002; Wu et al., 2004) or an inverse correlation between EphB4 receptor expression and tumor malignancy (Berclaz et al., 2002; Fox and Kandpal, 2004). By contrast, in Ha-Ras transgenic mice, both EphB4 and EphA2 are highly expressed in invasive breast tumors as compared with nonmetastatic tumors of C-myc transgenics (Andres and Ziemiecki, 2003), and the transgenic expression of EphB4 accelerated malignancy in Neu-T

transgenic mice (Munarini et al., 2002). EphA2 is upregulated in many invasive tumors, and it has been shown to be overexpressed in metastatic breast cancer (Ogawa et al., 2000; Fox and Kandpal, 2004). Although evidence supports a role for Eph receptors in breast cancer, the exact role and specific players are still unclear.

The EphB2 gene was recently implicated as a prostate cancer tumor suppressor gene. A screen of DNA samples obtained from uncultured, clinical prostate tumors, including 33 primary and 62 metastasis specimens, identified several cancer-associated mutations in EphB2 (Huusko et al., 2004). The mutational inactivation of EphB2 occurred in about 8% of primary and metastatic prostate cancers. To test whether EphB2 may represent a tumor suppressor, DU 145 cells (which do not express functional EphB2) were transfected with wild-type EphB2 constructs that suppressed the growth and colony formation of DU 145 cells, thus supporting the functional relevance of EphB2 mutations in prostate cancer progression (Huusko et al., 2004). It has also recently been reported that the K1019X mutation in the EphB2 gene differs in frequency between African Americans and Americans of European decent. This mutation is associated with an increased risk for prostate cancer, and it may be an important genetic risk factor for prostate cancer in African Americans (Kittles et al., 2006).

C. Eph Signaling in Cancer

It is not surprising that the Eph–ephrin system may play a significant role in metastasis and invasion, because these molecules normally function in cell–cell and cell–substrate adhesion as well as cell morphology and motility. Stimulating EphA3-expressing melanoma cell lines with clustered soluble ephrinA5 (but not monomeric ligand) resulted in a rapid reorganization of the actin and myosin cytoskeleton. This reorganization required the activation of RhoA that led to the retraction of cellular protrusions, membrane blebbing, and detachment. Furthermore, the adaptor CrkII is recruited to the EphA3 receptor in response to ephrinA5, and it is necessary for these RhoA-mediated responses (see Figure 22.1; Lawrenson et al., 2002). These and other studies suggest a possible role for integrin-mediated signaling. There are conflicting reports in the literature regarding the role of integrin signaling in cancer. For example, the activation of EphB1 has been associated with cell adhesion via integrins $\alpha v\beta 3$ and $\alpha 5\beta 1$ in teratocarcinoma cells (Huynh-Do et al., 1999), whereas EphA2 activation transiently inhibits integrin-mediated cell–substrate adhesion and results in FAK dephosphorylation in prostate carcinoma cells (Miao et al., 2001). By contrast, another study found that the ephrinA1 stimulation of prostate carcinoma cells overexpressing EphA2 led to FAK phosphorylation and increased cell–substrate adhesion (Carter et al., 2002). In yet another study, the prominent expression of ephrinB2 was observed in the invasive front of advanced malignant melanoma (Meyer et al., 2005). The overexpression of ephrinB2 in a mouse malignant melanoma line was shown to cause the formation of multiple lamellipodia, the constitutive activation of FAK, and a significant increase of $\beta 1$-integrin–mediated attachment to matrix components. Furthermore, ephrinB2 overexpression led to the enhanced activities of these cells in invasion experiments and cell migration assays, suggesting that signaling from this protein may contribute to the expansion and metastatic spread of malignant melanoma *in vivo*.

R-Ras, which is a member of the Ras family of GTPases, has been shown to positively affect integrin-mediated adhesion, and it has also been implicated in Eph receptor signaling (Pasquale, 2005). The negative regulation of R-Ras has been associated with the activation of EphB2 in fibroblasts and embryonal kidney cells in culture (Zou et al., 1999). This negative regulation occurs through the phosphorylation of the effector domain, but recently it has also been shown that the activation of both EphA and EphB receptors leads to a reduction in R-Ras activity through phosphorylation and GTP hydrolysis via p120RasGAP (see Figure 22.1; Dail, 2006). The mode of R-Ras regulation has differing effects on COS cells in culture; whereas guanosine triphosphate activating protein (GAP) activity blocks periphery retraction, R-Ras phosphorylation is required for the inhibition of cell migration (Dail et al., 2006). In human brain tumor specimens, EphB2 was overexpressed, and this led to a loss of cell adhesion and increased invasion. Upon EphB2 activation, R-Ras associated with the receptor and became highly phosphorylated. The depletion of endogenous R-Ras inhibited the EphB2 effects on glioma cell adhesion, proliferation, and invasion in *ex vivo* rat brain slices (Nakada et al., 2005). As discussed previously, Eph receptors and ephrins have significant links to the Rho family of small GTPases, which are regulators of actin dynamics, cell motility, cell–cell and cell–extracellular matrix adhesion, and cell-cycle progression. Each of these functions plays a critical role in the development and progression of cancer, and Rho family GTPases are frequently overexpressed in many cancers (Noren and Pasquale, 2004; Fritz and Kaina, 2006).

A recent study by Noren et al. (2006) has revealed a role for the tyrosine kinase ABL and the adaptor protein Crk in breast tumorigenesis. However, this study also has implications regarding the use of Gleevec (imatinib mesylate), a small-molecule inhibitor of the oncogenic BCR-ABL, which is used as an effective treatment for chronic myelogenous leukemia. It was found that EphB4 was expressed in non-tumorigenic and tumorigenic mammary cell lines, but only the non-tumorigenic MCF10A cells displayed a high level of the EphB ligand ephrinB2. In breast cancer cells, EphB4 receptor can be stimulated with a soluble ephrinB2 fused to an Fc domain of immunoglobulin. Intravenous administration of ephrinB2-Fc reduced tumor burden in mice xenografts of human breast cancer cells. *In vitro*, activation of EphB4 by ephrinB2-Fc reduced DNA synthesis, induced apoptosis, and inhibited cell migration and invasion. The authors further found that the adaptor protein Crk is phosphorylated by the tyrosine kinase ABL in response to EphB4 activation. Inhibiting expression or activity of Crk or ABL prevented the tumor suppression by ephrinB2-Fc in breast cancer cells. Of particular importance, Gleevec also blocked the tumor suppression mediated by ephrinB2-Fc, but a Gleevec resistant form of ABL circumvented this block to tumor suppression in mouse xenografts. Thus, ABL plays a major role in Eph-receptor-dependent tumor suppression (Noren et al., 2006).

Eph–ephrin modulation of cell–cell adhesion may also involve cadherin, a protein that is generally expressed at lower levels in tumor cells that have higher rates of metastasis (Widelitz, 2005). Cadherins are calcium-dependent adhesion molecules that either possess transmembrane domains or that are membrane bound by phospholipids anchors. Cadherins often enhance cell–cell adhesion through interactions between their extracellular domains on two juxtaposed cells. They can influence signal transduction by interacting

with coreceptors on the cell surface or by binding the intracellular domain to α-catenin and β-catenin. Cadherins assist with the establishment of cell polarity and tight junctions, and they are integral players in cell sorting (Widelitz, 2005). The inhibition of cadherin activity by either neutralizing antibodies or dominant-negative forms of cadherins leads to enhanced metastasis, whereas rescuing cadherin expression blocks this process (Widelitz, 2005).

Cadherin has been shown to rescue the EphA4- or ephrinB1-induced loss of cell–cell contact (Winning et al., 1996; Jones et al., 1998). The overexpression of EphA2 protein in colorectal and urinary bladder carcinoma tissue correlates closely with cancer progression and metastasis, which display an inverse correlation with E-cadherin expression. Thus, both EphA2 and E-cadherin may play an important role in tumor metastasis in colorectal cancer (Saito et al., 2004; Abraham et al., 2006). A fertile area for future research is likely to be found in studies designed to understand the precise nature of the coordinated regulation between Eph receptors or ephrins and cadherins.

The MAPK pathway is activated by a plethora of receptor tyrosine kinases, and it plays a key role in proliferation, differentiation, cell–substrate adhesion, and motility (Yoon and Seger, 2006). EphA2 receptors have the distinction of either activating or inhibiting MAPK, depending on the cellular context (Poliakov et al., 2004). These differences may be the result of the specificity of the association between the Eph receptor or ligand with the inhibitors (i.e., Ras-GAP, phosphorylated R-Ras) or activators (Grb2-SOS) of the MAPK pathway. In breast cancer cell lines, a positive effect of EphA2 on MAPK activation was found that led to reduced cell–substrate adhesion (Pratt and Kinch, 2002); however, in prostate cancer cells, the opposite was observed (Miao et al., 2003).

The intracellular signaling pathways activated and repressed by Eph receptors and ephrins are only beginning to emerge, and it is still unclear which specific signaling events mediated by these molecules are critical for cancer progression. The nature of Eph–ephrin signaling, the broad range of functions affecting cell movement and morphology, and the cross-talk with other signaling pathways (e.g., members of the Wnt, platelet-derived growth factor, and FGF pathways) that play roles in cell survival, angiogenesis, and motility in tumors make them appealing but enigmatic therapeutic targets.

D. Eph Receptors and Ephrins as Therapeutic Targets

Agents that target specific receptor tyrosine kinases at the cell surface have shown that, in specific cancers, appropriately targeted inhibitors can be efficacious and that they can demonstrate reduced toxicity as compared with standard chemotherapeutic agents. Because Eph–ephrin signaling requires cell–cell contact, an opportunity exists to use soluble forms of either the ligand or the receptor that bind but do not activate signaling (see Figure 22.4). Soluble EphA2 or EphA3 receptors have been tested in the pancreatic islet cell carcinoma mouse model in which SV40 large T antigen is expressed in pancreatic islet cells under the control of an insulin receptor. Premalignant angiogenic islets were inhibited along with a demonstration of reduced carcinoma tumor volume (Cheng et al., 2003). Similar reductions in tumor angiogenesis and tumor volume were demonstrated in other mouse studies using a metastatic mammary carcinoma model (Brantley et al., 2002) or tumors derived from a human pancreatic carcinoma line (Dobrzanski et al., 2004). Small interfering RNAs targeting EphA2 have been also tested by intravenous administration

into a xenograft model of pancreatic ductal carcinoma. This therapy was nontoxic, and it showed an efficient reduction of EphA2 expression and the concomitant inhibition of tumor growth and metastasis (Duxbury et al., 2004). In another study, a liposome-encapsulated EphA2 siRNA was highly effective for reducing *in vivo* EphA2 expression; in an orthotopic mouse model of ovarian cancer, it was shown to reduce tumor growth (Landen et al., 2005).

Human adenovirus vectors expressing secretory forms of ephrinA1 were used for the *in vivo* targeting of EphA2-overexpressing mammary tumors. The soluble ligand induced EphA2 activation and turnover in mouse mammary cancer cell lines, thereby leading to the inhibition of tumor formation when these lines were introduced into mice. Moreover, tumor growth was slowed by the intratumoral inoculation of soluble ligand into mice bearing the EphA2-overexpressing tumors (Noblitt et al., 2005). A slightly different strategy was also used that had a similar mechanism of action; the delivery of an activating monoclonal antibody *in vivo* inhibited tumor growth in a mammary tumor cell xenograft model (Coffman et al., 2003).

Monoclonal antibodies have also been generated to the extracellular sequence of EphB2, and they have been shown to effectively block the interaction of EphB2 with ephrin ligands and to inhibit the resulting autophosphorylation of the receptor (Mao et al., 2004). However, this antibody did not affect the proliferation of cancer cells expressing EphB2. Because this antibody was rapidly internalized after binding EphB2, it was conjugated to monomethylauristatin E. In this form, it specifically killed EphB2-expressing cancer cells *in vitro* and *in vivo* (Mao et al., 2004).

Soluble monomeric EphB4-expressing melanoma cells have been generated to test the effect of dominant-negative soluble EphB4 on tumor growth and angiogenesis. Soluble EphB4-expressing tumors grown subcutaneously in nude mice show dramatically reduced tumor growth as compared with control tumors. In addition, a reduction of intratumoral microvessel density was observed that corresponded with a matched-pair analysis of EphB4 and ephrinB2 expression in human colon carcinomas, and this revealed significantly upregulated levels of EphB4 expression as compared with adjacent normal tissue. Taken together, the data identify therapeutic effects on both tumor growth and vascularization (Martiny-Barony et al., 2004).

Another therapeutic approach is derived from the generation of EphA2 mimetic peptides that target the ligand-binding domain of EphA2 (Koolpe et al., 2002; Alves et al., 2003). These peptides compete with ephrins for binding to the receptor. One of these peptides was shown to be of possible use in immunotherapy. Mouse cytotoxic T lymphocytes raised against the EphA2 peptide showed specific tumor-killing ability for EphA2-expressing cancers, and specific EphA2 epitopes were able to be recognized on tumors of various origins (i.e., renal cell, lung, and colon carcinoma and sarcoma) from humans (Alves et al., 2003). Peptides that can selectively bind to different EphB class receptors and thus compete with ephrinB binding have also been identified (Koolpe et al., 2005; Chrencik et al., 2006).

It is increasingly apparent that deregulated Eph–ephrin signaling that leads to a loss of cell–cell adhesion and repulsion and that other mechanisms that result in enhanced cell–cell and cell–substrate adhesion may all be involved in tumor invasion, metastasis, and angiogenesis. Other efforts targeting downstream members of the Eph–ephrin signaling pathways may also yield valuable therapeutic results. However, a detailed understanding of these

pathways will be critical for devising therapeutic strategies to intercede in the prometastatic influence of the Eph–ephrin system.

E. Other Human Diseases

To date, the only known mutation in the ephrin–Eph receptor signaling system in humans is within ephrinB1, and this leads to a morphogenetic disorder called *craniofrontonasal syndrome* (CFNS), which is an X-linked developmental disorder that shows greater severity in heterozygous females than in hemizygous males (Twigg et al., 2004). Females have frontonasal dysplasia and fusion of the coronal sutures, whereas males present only with hypertelorism (i.e., excessive width between the eyes). The female craniofrontonasal syndrome phenotype is caused by heterozygous loss-of-function mutations in ephrinB1. Although ephrinB1 is X-inactivated, the study suggests that, in heterozygous females, the patchwork loss of ephrinB1 disturbs tissue boundary formation at the developing coronal suture; alternatively, in males who are deficient in ephrinB1, an alternative mechanism maintains the normal boundary (Twigg et al., 2004).

Davy et al. (2006) have shown that ephrinB1+/- mice exhibit calvarial defects, a phenotype that is very similar to CFNS and correlates with cell sorting defects in neural crest cells (Davy et al., 2006). These defects were found to result from impaired differentiation of osteogenic precursors. Moreover, they show that gap junction communication (GJC) was inhibited at ectopic ephrin boundaries and that ephrinB1 interacts with Connexin43 (Cx43) and regulates its distribution. Cx43 is one of several Gap junctional proteins that regulate the diffusion or transmission of specific second messengers and metabolites among cells. EphrinB1 and its receptor EphB2 were shown to inhibit gap junction communication in cell culture and that over-expression of Cx43 partially rescues the facial defects in ephrinB1 mutants. Collectively, these findings indicate that the facial abnormalities observed in CFNS may be caused by reduced gap junction activity at ectopic Eph/ephrin boundaries (Davy et al., 2006).

Craniosynostosis affects 1 in every 2500 births, and many of the previously identified mutations result in the constitutive activation of FGF receptor family members 1, 2, and 3 or result from single mutations in the transcription factors TWIST or MSX2 (Wilkie, 2005). These studies are particularly interesting in the light of the interactions between the ephrinB1 and the FGF receptor signaling pathways that have been reported (Chong et al., 2000; Moore et al., 2004), which suggests a possible mechanism by which boundary formation might lead to the control of the coronal suture formation (Twigg et al., 2004). The exact mechanism and role of the interaction between ephrinBs and FGF receptors in pathobiology await further study. Moreover, further studies are needed to reveal whether mutations in other Eph–ephrin family members are implicated in human disease.

SUMMARY

- Interactions between the Eph receptor tyrosine kinases residing on one cell with their membrane-bound ligands on another cell result in bidirectional signaling.

- Although evidence is emerging that both Eph receptors and ligands ultimately affect Rho family signal transduction, various signaling molecules and pathways intersect with Eph receptor or ligand signaling, and further studies are needed to define the Eph–ephrin signal transduction systems.
- Eph–ephrin signaling from cell–cell contact events during development leads to cell sorting and boundary formation between receptor- and ligand-bearing cells.
- Motile ligand- or receptor-bearing cells respond to contact with cells bearing the cognate receptor or ligand by adhesion or repulsion.
- Alternative growth factors and signaling pathways can mediate or regulate Eph–ephrin signaling to assist the cognate receptor- or ligand-bearing cells to regulate the movement and positioning of cells.
- These ligands and receptors play a role in several morphogenetic events during development; however, when they are deregulated, they can lead to cancer invasion and metastasis.
- Because Eph–ephrin signaling requires cell–cell contact, these cell-surface proteins represent amenable therapeutic targets for soluble binding or interfering molecules to prevent intracellular signaling.

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GLOSSARY

Coronal suture
The suture (the fibrous joint between the bones of the skull) extending across the skull between the two parietal bones and the frontal bone.

Craniosynostosis
The premature fusion of the cranial sutures during embryonic development that results in an abnormal shape and growth of the head.

Eph
Erythropoietin-producing hepatoma; this refers to the origin tissue of the Eph receptor tyrosine kinases.

Ephrins
Eph family *r*eceptor *in*teracting proteins.

Frontonasal dysplasia
Also called *median cleft syndrome;* a disorder resulting in the malformation of the central portions of the face and head.

Morphogenesis
The development, through growth and differentiation, of form and structure in an organism.

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IV

ECTODERMAL ORGANS

23 NEURAL CELL FATE DETERMINATION

STEPHEN N. SANSOM and FREDERICK J. LIVESEY

Gurdon Institute and Department of Biochemistry, University of Cambridge, Cambridge, United Kingdom

INTRODUCTION

The mature nervous system in any organism contains many different distinct types of neurons as defined by their morphology, connectivity, neurotransmitter phenotype, and electrophysiologic properties. Neurogenesis, which is the process by which postmitotic neurons are generated from pools of mitotic progenitor cells, is a highly regulated process (Edlund and Jessell, 1999; Livesey and Cepko, 2001). Different types of neurons are produced in a temporal sequence that is conserved in different species, and different types of neurons are produced in different parts of the nervous system (Cepko et al., 1996b). Discrete phenotypes or identities are assigned to the postmitotic progeny of neural progenitor cells through a process of cell fate determination. To a significant degree, the fates of those progeny are decided within the mitotic progenitor cell before it divides. Thus, progenitor cells have an integrative function whereby they combine extrinsic information in the form of extracellular signals with information intrinsic to the cell to decide the fates of their daughter cells; this will be discussed in more detail later in this chapter.

The developmental biology of neural cell fate determination can be broadly divided into a series of processes: the induction or appearance of neurogenic tissue(s) (i.e., tissue-containing neural stem and progenitor cells; see Chapter 12); the division of this tissue into distinct territories or regions that go on to form different components of the adult nervous system; and the ordered production of region-specific neurons within each territory. Several striking recent studies have clearly shown that this process can be recapitulated *in vitro* by generating particular classes of neurons from embryonic stem cells through a series of discrete steps aimed at guiding cells through each stage in this process (Kim et al., 2002; Wichterle et al., 2002). In this chapter,

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we review the principles by which neural cell fates are determined using selected examples to highlight fundamental processes and concepts.

I. THE FUNDAMENTAL PROCESS OF NEUROGENESIS: PRONEURAL GENES

A. Proneural Gene Families in Flies and Vertebrates

Proneural genes are transcription factors which contain a basic helix–loop–helix (bHLH) domain that confers dimerization and DNA binding properties (Murre et al., 1989). The proneural genes were originally identified in Drosophila during the early 1970s as a complex of genes involved in the early stages of neural development (Garcia-Bellido, 1979; Ghysen and Dambly-Chaudiere, 1988). Proneural genes are both necessary and sufficient to initiate the development of neuronal lineages and to promote the generation of progenitors that are committed to neuronal differentiation.

Two classes of proneural gene are known in Drosophila. The *achaete-scute (asc)* family consists of four genes: *achaete, scute, lethal of scute,* and *asense* (Gonzalez et al., 1989; Villares and Cabrera, 1987). The second, the *atonal (ato)* family, has three members, *atonal, amos,* and *cato* (Goulding et al., 2000a; 2000b; Huang et al., 2000b; Jarman et al., 1993). In vertebrates, there are several families of proneural genes that are named according to their homology with those in Drosophila: these are the *achaete–scute* homologs *(ash)*, the *atonal* homologs *(ath)*, and the *atonal*-related gene families (Guillemot, 1999; Lee, 1997). The vertebrate *ash* family consists of four members *(ash1* through *ash4)*, which are prefixed in vertebrates by the first letter of the species name: for example, *ash1* in mice is *Mash1;* in *Xenopus,* it is *Xash1;* and in zebrafish, it is *Zash1*. The vertebrate *ath* gene family is larger, but only two of its members are considered true orthologs of the Drosophila *ato* genes (these are *Math1* and *Math5* in mice). Examples of the vertebrate *atonal*-related families are the *NeuroD, Neurogenin,* and *Olig* gene families (Hassan and Bellen, 2000; Lee, 1997). These family relations are based on the presence of specific residues within the bHLH domain.

B. How Do Proneural Genes Function?

Proneural genes function by binding to DNA as heterodimers with the ubiquitously expressed bHLH "E" proteins: E2A, HEB, and E2–2 in vertebrates and *daughterless (da)* in Drosophila (Cabrera and Alonso, 1991; Johnson et al., 1992; Massari and Murre, 2000). The bHLH domain of the proneural genes contains a stretch of 10 DNA binding residues of which nine are conserved among all proneural genes (Bertrand et al., 2002; Chien et al., 1996). These conserved DNA binding residues recognize the E-box (CANNT) promoter element. Most proneural genes function as activators of target gene transcription, with the exception of *Olig2,* which is a repressor (Cabrera and Alonso, 1991; Johnson et al., 1992; Mizuguchi et al., 2001; Novitch et al., 2001). The repression of proneural function can be achieved by the disruption of their heterodimerization with the ubiquitous E proteins. The Drosophila *extra macrochaetae (emc)* and the vertebrate *inhibitor of differentiation (Id)* genes possess bHLH domains, but they lack DNA binding motifs, and they are thought to compete with proneural proteins for E proteins, thus inhibiting proneural gene function (Cabrera and Alonso, 1991; Campuzano, 2001;

Yokota, 2001). The Drosophila *hairy* and *enhancer of split (Espl)* and the vertebrate *hairy and enhancer of split* homolog *(Hes), hairy and enhancer of split related (Her),* and *enhancer of split related (Esr)* genes are transcriptional repressors of proneural genes, and they are also thought to repress proneural function by the disruption of heterodimer formation (Davis and Turner, 2001; Kageyama and Nakanishi, 1997).

C. Proneural Genes Specify Neural Progenitor Cells

In Drosophila, a major role of the proneural genes is to promote the specification of neural progenitors in both the peripheral nervous system (PNS) and the central nervous system (CNS). Mutations that disrupt proneural gene function in Drosophila result in a reduction in the numbers of neural progenitors generated, whereas the overexpression of proneural genes results in the ectopic formation of neural progenitor cells (Dominguez and Campuzano, 1993; Jimenez and Campos-Ortega, 1990; Rodriguez et al., 1990). In vertebrates, the *ash, atoh,* and *ngn* genes have a proneural role that is similar to the role of their Drosophila homologues. The loss of neural progenitors in vertebrate models that are mutant for proneural gene function is correlated with premature astrocyte generation, and there is evidence that proneural genes promote the neural fate and repress the glial fate in vertebrates (Casarosa et al., 1999; Cau et al., 2002; Fode et al., 1998; Guillemot and Joyner, 1993; Horton et al., 1999; Ma et al., 1998, 1999; Scardigli et al., 2001).

Other vertebrate proneural genes (e.g., *NeuroD, Math3/NeuroM*) have characteristics that are more similar to those of neural differentiation genes, but that are also implicated in dictating a neuronal rather than a glial cell fate choice in some regions (Morrow et al., 1999; Tomita et al., 2000). As is the case in Drosophila, the overexpression of many vertebrate proneural genes results in the promotion of neuronal differentiation, while the opposite phenotype is observed in loss-of-function studies (Blader et al., 1997; Ma et al., 1996; Mizuguchi et al., 2001). However, direct evidence for the proneural function of some vertebrate proneural genes is lacking. For example, *Math1* and *Math5* are involved in specifying neuronal identity, but they do not seem to have a proneural function (Bermingham et al., 1999; Gowan et al., 2001; Hassan and Bellen, 2000). Mutational studies in the mouse have only established classic proneural function for a few genes, including *Mash1, Ngn1,* and *Ngn2*. Furthermore, the known vertebrate proneural genes do not account for the generation of all of the known neural lineages (Fode et al., 1998; Ma et al., 1998; Ma et al., 1999; Sommer et al., 1995). There are, therefore, many similarities, but also clear differences in the roles of the proneural genes with regard to progenitor cell selection in vertebrates and flies.

D. The Role of Proneural Genes in Neuronal Differentiation

After selection, neural progenitor cells further upregulate proneural gene expression before becoming committed to differentiation (Culi and Modolell, 1998; Kintner, 2002; Koyano-Nakagawa et al., 1999; Vaessin et al., 1994). Positive feedback loops serve to maintain and upregulate proneural gene expression in prospective progenitor cells. For example, the transcription factors *senseless* in Drosophila and *Xcoe2* and *Hes6* in vertebrates are induced by proneural genes, and upregulate proneural gene expression (Bae et al., 2000; Dubois et al., 1998; Koyano-Nakagawa et al., 2000; Nolo et al., 2000). Some proneural genes are subject to autoregulation, such as the vertebrate

atonal homolog, Math1; while conversely, other vertebrate proneural genes, such as *Mash1* and *Ngn1*, do not directly regulate their own expression (Guillemot et al., 1993; Helms et al., 2000; Nieto et al., 2001; Sun et al., 1998; Van Doren et al., 1992).

Although the proneural genes have a role in the promotion of neural fate, proneural gene expression in neural progenitors is transient. In vertebrates, proneural genes are downregulated before progenitors exit the proliferative zone of the neural tube and begin to differentiate (Ben-Arie et al., 1996; Gradwohl et al., 1996; Ma et al., 1998). In Drosophila, proneural genes are downregulated before progenitors start to generate the sense organs of the PNS and the ganglion mother cells of the CNS (Cubas et al., 1991; Jarman et al., 1993; Skeath and Carroll, 1991). Therefore, proneural genes function to confer a neural fate by switching on downstream genes, which are known as the *neuronal differentiation genes.*

Many neuronal differentiation genes possess bHLH domains and are related to the proneural genes. This has given rise to the idea that cascades of different bHLH genes are responsible for neural cell fate determination and differentiation, as is the situation in muscle differentiation (Jan and Jan, 1993; Kintner, 2002; Lee, 1997; Weintraub, 1993). bHLH neuronal differentiation genes are expressed later than the proneural genes, are under the transcriptional control of proneural genes, and can promote neuronal differentiation if they are ectopically expressed. In the fly, *Asense* is a direct transcriptional target of *Achaete* and *Scute*, and is involved in sense-organ differentiation (Dominguez and Campuzano, 1993; Jarman et al., 1993). In vertebrates, bHLH genes of the *NeuroD* family are downstream of the neurogenins (Fode et al., 1998; Huang et al., 2000a; Ma et al., 1998), and have the characteristics of neural differentiation genes (Farah et al., 2000; Lee et al., 1995; Liu et al., 2000; Miyata et al., 1999; Olson et al., 2001; Schwab et al., 2000). Because proneural genes and neuronal differentiation genes are structurally related, it is plausible that their distinct functions may be the result of the different times at which they are expressed. This possibility has not been fully investigated, although there is evidence that several proneural genes control differentiation steps in certain neuronal lineages.

E. Proneural Genes Have a Role in the Specification of Neuronal Identity

In addition to their role in progenitor selection, a role for proneural genes in the specification of neuronal identity emerged. Proneural genes are often expressed in restricted progenitor domains that will give rise to particular types of neurons. In the dorsal vertebrate spinal cord, *Math1, Ngn1,* and *Mash1* are expressed in distinct dorsoventral progenitor domains that produce distinct types of interneuron (Gowan et al., 2001). Mutant analysis in the mouse has shown that *Math1* and *Ngn1* are necessary for the correct specification of some neural progenitor domains, thus further linking proneural gene expression to neural cell fate determination (Bermingham et al., 2001; Gowan et al., 2001). In Drosophila, loss-of-function studies have shown that different types of proneural genes are involved in both the formation of different types of sense organs (Huang et al., 2000b; Jarman et al., 1993; Jarman et al., 1994) and the formation of different types of neurons in the CNS (Parras et al., 1996; Skeath and Doe, 1996).

In vertebrates, a well-studied example of the role of the proneural genes in the specification of neuronal identity is the role of *Mash1* in the generation of

noradrenergic neurons. In the PNS, loss- and gain-of-function experiments have shown that *Mash1* acts together with the homeodomain protein *Phox2b* to induce the expression of *Phox2a,* a related homeobox gene, and *dopamine β-hydroxylase,* in the specification of noradrenergic neurons in the sympathetic ganglia (Goridis and Rohrer, 2002; Hirsch et al., 1998; Lo et al., 1998; Pattyn et al., 1999). By contrast, in the noradrenergic centres of the brain, *Mash1* induces the expression of both *Phox2b* and *Phox2a* (Goridis and Brunet, 1999; Pattyn et al., 2000). *Mash1* has also been implicated in the specification of other neuronal identities; for example, in the ventral forebrain, *Mash1* is expressed in domains that give rise GABAergic neurons (Fode et al., 2000; Parras et al., 2002). The involvement of *Mash1* in the specification of different kinds of neurons indicates that it must interact with regionally expressed factors that modify its specificity.

The vertebrate neurogenin genes are also thought to be involved in the specification of neuronal identity. In the PNS, a role has been established for the neurogenins in the specification of sensory neurons, and, in the CNS, *Ngn2* has been shown to cooperate with *Olig2* in motor neuron induction (Lo et al., 2002). In the retina, *NeuroD* and *Math3* are necessary and sufficient for the generation of amacrine interneurons (Inoue et al., 2002; Morrow et al., 1999), whereas *Math3* and *Mash1* are involved in specifying bipolar fate (Hatakeyama et al., 2001). The specification of neuronal fate can therefore be carried out by nonproneural bHLH proteins, and it is uncoupled from the selection of progenitors in some neural lineages.

In the mammalian neocortex, *Ngn1* and *Ngn2* are involved in both the spatial and temporal specification of neuronal identity. In this structure, *Ngn1* and *Ngn2* function to specify a glutamergic neuronal identity through the activation of cortical-specific pathways and by the repression of a subcortical GABAergic (subcortical) neuronal fate (Schuurmans et al., 2004). In addition to its proneural role in the developing neocortex, *Ngn2* is also required in this structure for specifying the migration properties and dendritic morphology of pyramidal neurons by a posttranslational mechanism (Hand et al., 2005).

Evidence from Drosophila indicates that different proneural genes regulate different target genes. For example, the gene *cut,* which is expressed in the progenitors of external sense organs, is induced by the *asc* genes, but it is repressed by *atonal* (Blochlinger et al., 1991; Jarman and Ahmed, 1998). Specificity in the regulation of target genes is thought to be conferred both by the different DNA binding properties of the different proneural genes and by regionally expressed cofactors. Sequence analysis of E-box motifs has revealed that different proneural proteins recognize distinct E-box sequences (Bertrand et al., 2002; Chien et al., 1996). In Drosophila, the regionally expressed cofactors Pannier and Chip have been identified, and they have been shown to modulate the Achaete/Scute-Daughterless–mediated activation of *achaete* transcription (Ramain et al., 2000).

II. SPATIAL CELL FATE DETERMINATION: MAKING THE RIGHT NEURON IN THE RIGHT PLACE

A primary event in the construction of a nervous system is the division of the nascent CNS into a number of discrete territories or regions, typically by conferring distinct regional identities on neural progenitor cells. A further round of the spatial patterning of progenitor cells then occurs within each region.

A. Regionalization of the Developing Vertebrate Spinal Cord

The primary regionalization event in the vertebrate CNS is its division into the broad territories of forebrain, midbrain, hindbrain, and spinal cord. Within each territory, a fine-scale regionalization of neural progenitor cells takes place. In vertebrates, the best understood example of fine-scale neural progenitor regionalization is found in the development of the neural tube (Figure 23.1). This structure is patterned dorsoventrally by molecules that are homologous to those responsible for patterning the ventral neurogenic region of the fly. The dorsoventral axis of the ventral half of the neural tube can be subdivided into five progenitor domains known as p0, p1, p2, MN, and p3 on the basis of differential gene expression (Briscoe et al., 2000). Each of these domains gives rise to a distinct class of neuron. Domains p0 through p3 give rise to V0 through V3 interneurons, whereas the pMN domain gives rise to motor neurons (Briscoe et al., 2000; Ericson et al., 1997a; Pierani et al., 2001; Sharma et al., 1998).

The five progenitor domains are initially specified by a gradient of the signaling molecule Sonic hedgehog (Shh), which is secreted from the ventral floor plate (Ericson et al., 1996; Roelink et al., 1995). The progenitors of the neural tube are highly sensitive to the ambient concentration of Shh, and this results in the graded expression of a group of transcription factors (spinal cord TFs) by the neural tube progenitor cells (Briscoe et al., 2001; Briscoe et al., 2000; Ericson et al., 1996). Many of the spinal cord TFs possess homeodomains, although one is a bHLH factor (*Olig2;* Lee and Pfaff, 2001). These spinal cord TFs can be divided into two classes. Class I factors (Pax6, Irx3, Dbx2, Dbx1, and Pax3/7) are repressed by Shh signaling, whereas class II factors (Nkx2.2/2.9, *Olig2,* Nkx6.1, and Nkx6.2) are induced by Shh (Briscoe et al., 2000).

Furthermore, class I and II spinal cord TFs are antagonistic, and they downregulate the expression of one another in a process known as *cross regulation,* which functions to establish sharp boundaries in gene expression

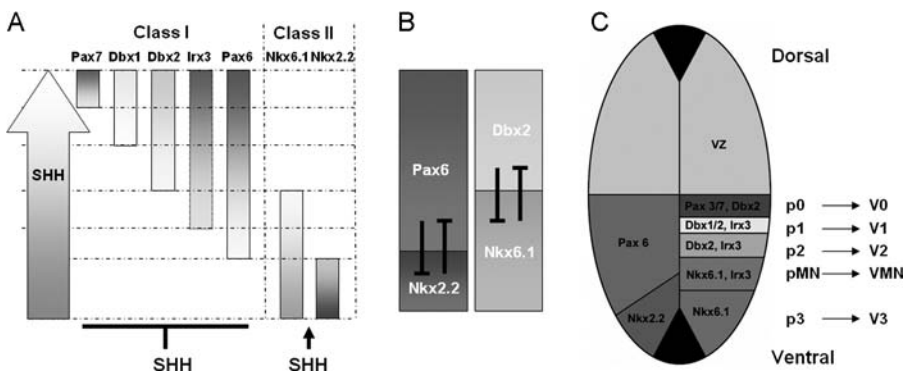

FIGURE 23.1 **Spatial control of cell fate determination in the developing spinal cord. A,** The secreted protein Sonic hedgehog (Shh) mediates the inhibition of class I homeodomain transcription factors and the induction of class II proteins at varying concentrations. **B,** Homeodomain proteins cross repress one another at a common progenitor domain boundary. **C,** Cross repression between class I and II proteins sets up five distinct progenitor domains (p0, p1, p2, pMN, and p3). These progenitor domains have been shown to act in a combinatorial manner to specify distinct neural identities (V0, V1, V2, and V3 represent interneurons; MN denotes motor neurons) within the spinal cord. *VZ,* Ventricular zone. (See color insert.)

(Briscoe et al., 2000; Ericson et al., 1997b; Mizuguchi et al., 2001; Novitch et al., 2001; Sander et al., 2000; Vallstedt et al., 2001). The expression of combinations of these spinal cord TFs defines the five progenitor domains (Jessell, 2000). Functional studies have demonstrated that these TFs act in a combinatorial manner to specify distinct neural identities (Briscoe et al., 2000; Briscoe et al., 1999; Ericson et al., 1997b; Mansouri and Gruss, 1998; Sander et al., 2000). Several of the spinal cord TFs are homologous to genes involved in the dorsoventral patterning of the Drosophila ventral neurogenic region: *nkx2.2* is related to *vnd, gsh-1/2* is related to *ind,* and *msx* is related to *msh(dr)* (Cornell and Ohlen, 2000).

The intracellular mechanisms by which spinal cord TFs are expressed in response to the Shh gradient are an area of active research. Members of the *Gli/ci* gene family, which is known to be downstream of Shh signaling in Drosophila, have been implicated in this process (Ding et al., 1998; Litingtung and Chiang, 2000; Matise et al., 1998). *Gli* levels mirror Shh levels in the neural tube, and a gradient of Gli activity can replace Shh signaling in dorsoventral patterning (Stamataki et al., 2005). It is also known that Shh is able to induce the expression of target genes via *Gli/ci* independent mechanisms (Krishnan et al., 1997).

B. How Do Spinal Cord Transcription Factors Function in the Specification of Neuronal Identity?

Contrary to expectation, spinal cord TFs are understood to act by the repression of their target genes. Eight of the 11 spinal cord TFs possess an Engrailed homology (eh1) domain that is conserved with the engrailed repressor (Muhr et al., 2001; Smith and Jaynes, 1996). This domain is understood to interact with the Groucho-TLE (Gro/TLE) corepressors, which are broadly expressed in the developing neural tube (Allen and Walsh, 1999; Muhr et al., 2001). The Gro/TLE repressors are thought to mediate gene regulation by promoting interaction with histone deacetylases to modulate chromatin structure or possibly by more direct interaction with the transcription machinery (Chen et al., 1999; Edmondson and Roth, 1998; Edmondson et al., 1996; Lee and Pfaff, 2001). It is possible that additional corepressors function with spinal cord TFs in the developing neural tube.

Thus, spinal cord TFs are understood to act to confer neural fate by the negative regulation of their target genes. Differential target gene expression in the five progenitor regions might be achieved by the presence of different TF binding sites in the promoters of different target genes (Lee and Pfaff, 2001). In this model, the downstream target genes of the spinal cord TFs are initially broadly expressed, but then become restricted to permissive progenitor domains. Examples of targets of spinal cord TFs are *Evx1* in VO cells, *En1* in V1 cells, *Lhx3/4* and *Chx10* in V2 cells, MNR2/HB0 and *Isl1/2* in motor neurons, and *Sim1* in V3 interneurons (Lee and Pfaff, 2001). The expression of these downstream genes begins in the spinal cord neurons when they exit the cell cycle, and is responsible for conferring specific neuronal phenotypes (Lee and Pfaff, 2001). Studies of mouse mutant models of the spinal cord TF target genes have revealed that, although some of these genes dictate all aspects of cell identity, others only act to specify certain features of cell fate, such as axon guidance properties (Matise and Joyner, 1997; Moran-Rivard et al., 2001; Saueressig et al., 1999).

C. Conferring Rostrocaudal Positional Identities

The rostrocaudal patterning of neural progenitor cells in the spinal cord is not as well understood as their dorsoventral patterning. Although most kinds of neuron are represented at the different segmental levels of the spinal cord, strikingly, some classes of motor neurons are not (Jessell, 2000). Grafting experiments have shown that the initial rostrocaudal patterning of the neural tube is carried out by interactions with the paraxial mesoderm (Appel et al., 1995; Ensini et al., 1998; Itasaki et al., 1996; Lance-Jones et al., 2001; Lumsden and Krumlauf, 1996). Although the signals involved in this patterning are still being elucidated, signaling by retinoic acid is known to be important (Muhr et al., 1999) and has been shown to control rostrocaudal identity in postmitotic motor neurons (Sockanathan et al., 2003).

A class of genes understood to be important for the rostrocaudal patterning of neurons in the spinal cord is the classical *Homeobox (Hox)* genes. There are four *Hox* gene clusters: a, b, c, and d in vertebrates, which are related to the homeotic genes of Drosophila (see Chapter 9). In vertebrates, *Hox* genes have a well-established role in axial patterning (Burke et al., 1995). Members of the *Hox-c* and *Hox-d* gene clusters are expressed at different rostrocaudal levels of the spinal cord, and there is evidence that they are necessary for the specification of some classes of neuron in the developing neural tube, which indicates that *Hox* genes play a role in the rostrocaudal regionalization of the nervous system (Belting et al., 1998; de la Cruz et al., 1999; Keynes and Krumlauf, 1994). In support of this, several classes of *Hox* genes have been shown to control motor neuron columnar identity as well as motor pool identity and motor-neuron–target-cell connections (Dasen et al., 2003; 2005).

In the developing neocortex, neurons of the cortical plate are regionally specified into a classic pattern of neocortical areas (Mountcastle, 1998). In this structure, these distinct neuronal domains are generated by neocortical neural progenitor cells, which carry an intrinsic spatial pattern known as the *neocortical protomap* (Rakic, 1988). Initially specified by the action of secreted factors, such as fibroblast growth factors, the neocortical protomap is manifest as gradients of TF expression across the field of neocortical progenitor cells (Bishop et al., 2000; Mallamaci et al., 2000; Sansom et al., 2005). The transcription factor Emx2 is expressed in a high caudolateral to low rostromedial gradient in neocortical progenitor cells, and both loss- and gain-of-function studies have demonstrated the importance of this TF gradient in the spatial specification of neuronal identity (Bishop et al., 2000; Hamasaki et al., 2004; Mallamaci et al., 2000). Thus, in the neocortex, the generation of spatially distinct neuronal progeny is apparently not preceded by the formation of distinct progenitor domains as is the case elsewhere in the developing nervous system (reviewed by Chambers and Fishell, 2006; Mallamaci and Stoykova, 2006).

D. Progenitor Cells Within a Given Domain Are Multipotent

An important feature of the progenitor domains in the developing spinal cord is that, although they may be defined by the expression of regional factors and proneural genes, these domains are not restricted to the generation of a single type of neuron. In the pMN domain of the ventral vertebrate spinal cord, progenitors are known to undergo a switch from motor neuron production to

oligodendrocyte generation over time (Lu et al., 2000; Pringle et al., 1998; Richardson et al., 1997; Soula et al., 2001; Zhou et al., 2000; 2001). In addition to temporal changes, it is possible that there is heterogeneity within individual progenitor domains.

III. TEMPORAL CONTROL OF CELL FATE DETERMINATION: MAKING THE RIGHT NEURON AT THE RIGHT TIME

The generation of the individual neurons of a specific neural lineage over time from a single multipotent progenitor cell presents a new problem. How does a neural progenitor cell give rise to a series of neurons with distinct identities over time? A key mechanism used in all nervous systems studied is the asymmetric division of stem and progenitor cells.

A. Asymmetric Cell Division in Drosophila

In Drosophila, both the CNS progenitors (neuroblasts) and the PNS progenitors (sensory organ precursors) undergo a series of asymmetric divisions to generate the characteristic lineages of neurons and glia (Bossing et al., 1996; Gho et al., 1999; Reddy and Rodrigues, 1999; Schmid et al., 1997; 1999). After delaminating from the neuroectoderm, Drosophila neuroblasts undergo a series of apical/basal orientated asymmetric divisions (Figure 23.2). These asymmetric divisions give rise to a smaller daughter cell, the ganglion mother cell (GMC), which buds off from the dorsal/lateral cortex of the neuroblast. GMCs then divide terminally to give rise to two neurons or glia.

The asymmetric division of neuroblasts requires the asymmetric localization of cell fate determinants and the correct orientation of the mitotic spindle for the proper segregation of cell fate determinants to the GMC daughter cell. The polarity of neuroblasts is established by an apical protein complex consisting of Bazooka, DaPKC, and DmPar6, which also mediates polarity in the epithelium (Petronczki and Knoblich, 2001; Schober et al., 1999; Wodarz et al., 2000; Wodarz et al., 1999). The cell-fate determinants Prospero, *prospero* mRNA, and Numb and the adapter molecules that help to localize them (Miranda, Staufen, and Partner of numb, respectively) form a basal crescent within the neuroblast (Broadus et al., 1998; Hirata et al., 1995; Ikeshima-Kataoka et al., 1997; Knoblich et al., 1995; Li et al., 1997; Lu et al., 1998; Rhyu et al., 1994; Schuldt et al., 1998; Shen et al., 1997; Spana and Doe, 1995). This basal crescent, which overlies the basal spindle pole of mitotic neuroblasts, segregates to the GMC daughter cell. The neural lineages of the peripheral nervous system are generated by the sensory organ precursors, which undergo a series of asymmetric cell divisions to give rise to four different cell types; together, these constitute an external sense organ (Bodmer et al., 1989).

B. Asymmetric Cell Division in Vertebrates

In vertebrates, the asymmetric cell division of neural progenitors has been reported in the cortex and the retina of the rat (Cayouette et al., 2001a; Chenn and McConnell, 1995). In the mammalian cerebral cortex, neural progenitors of the ventricular zone (VZ) give rise to the outer radial layers, which are composed of differentiated neurons that possess distinct identities. This process of layer formation in the cerebral cortex is known to involve

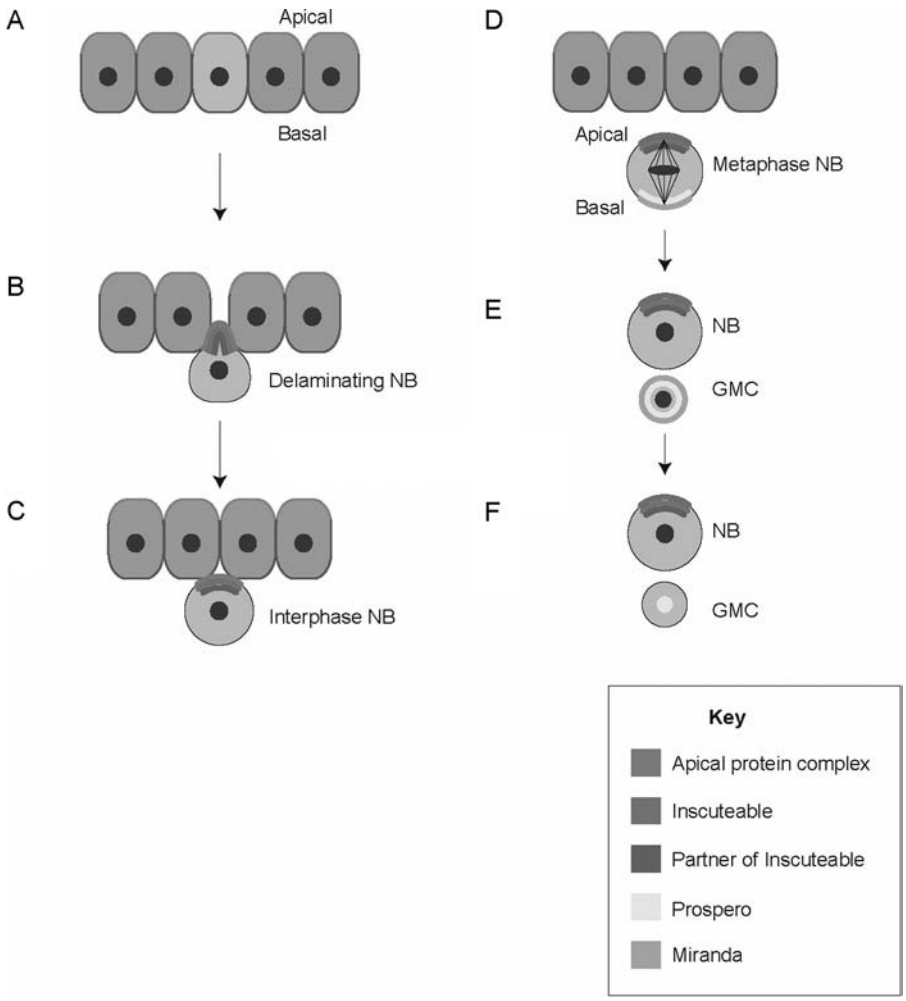

FIGURE 23.2 **Asymmetric division of neuroblasts in Drosophila. A,** The neuroblast is specified in the neuroectoderm. Polarity is determined in the neuroblast and the epithelium by a conserved apical protein complex *(green)* that consists of Bazooka, DmPar6, and DaPkC. Partner of Inscuteable (Pins) *(blue)* is cortically localized. **B** and **C,** In the interphase delaminating neuroblast, Inscuteable expression begins, and Inscuteable *(red)* binds to Bazooka, thus localizing Pins apically. **D,** In the delaminated neuroblast, a basal crescent forms that consists of Miranda *(pink)*, which binds to Prospero *(yellow)* and Staufen, and Partner of numb, which binds Numb (not shown). **E,** The neuroblast divides asymmetrically, producing a smaller daughter cell, the ganglion mother cell (GMC), and regenerating the neuroblast. The basal crescent of the neuroblast is segregated to the GMC. **F,** In the GMC, Miranda is rapidly degraded, and Prospero translocates to the nucleus, where it specifies a neural cell fate in this lineage. (See color insert.)

the asymmetric division of neural progenitor cells in the VZ, and this is followed by the outward migration of postmitotic neurons. It is thought that the formation of the different radial layers is the result of changes in and the restriction of progenitor cell competence in the VZ over time (Desai and McConnell, 2000).

The mouse homologue of the Drosophila cell fate determinant Numb, m-Numb, is known to be involved in the asymmetric divisions of the progenitors of the cerebral cortex (Zhong et al., 1996) and the retina (Cayouette et al., 2001b), and it is capable of rescuing *numb* mutant flies (Zhong et al.,

1996). In cultures of cortical progenitor cells, m-Numb has been shown to preferentially localize to the postmitotic cell in progenitor–neuron divisions, and, by comparison, m-Numb inheritance is unbiased in progenitor–progenitor divisions (Shen et al., 2002). Recent videomicroscopy analysis of retinal progenitor explants has demonstrated that the asymmetric inheritance of Numb between two retinal daughter cells promotes a different fate for each daughter, whereas the symmetric inheritance of Numb tends to lead to the same fate for both daughter cells (Cayouette and Raff, 2003). These observations indicate that there is conservation of Numb function in the developing nervous systems of Drosophila and vertebrates and that the generation of neural lineages by a series of asymmetric divisions is a common feature of neurogenesis.

Live imaging and time-lapse studies of neocortical neurogenesis and stem cell behaviors have discovered a hitherto unappreciated importance of a second population of neocortical progenitor cells, the subventricular zone (SVZ) or intermediate zone (IZ) progenitor cells (Haubensak et al., 2004; Noctor et al., 2004). The SVZ was generally held to be a minor structure in rodents as compared with primates, and it was thought that it generated some neurons but mainly glial cells late in development (Martinez-Cerdeno et al., 2006). A number of studies have unexpectedly found that the SVZ is composed of neurogenic progenitor cells early in development and that it contributes large numbers of neurons to the rodent cortex. SVZ cells are themselves generated by classical radial glia cells, the VZ cells that were proposed to be the primary neurogenic stem and progenitor cells within the cortex. The emerging model is that VZ stem and progenitor cells generate three types of cells: layer-specific neurons, IZ or transit-amplifying cells, and, in self-renewing divisions, more VZ cells.

C. Temporal Aspects of Neural Cell Fate Determination in the Vertebrate Retina

In the vertebrate retina, the initial steps of neural cell fate determination are remarkably similar to those seen in Drosophila. The first-born neurons are retinal ganglion cells, and their production requires the expression of the *atonal* homolog, *ath5*, which (as in the Drosophila retina) is induced by Shh and opposed by a gradient of Notch signaling (Neumann and Nuesslein-Volhard, 2000). In the vertebrate retina, six types of neuron and one type of glial cell are generated during development. The order in which these cell types appear is invariant across vertebrate species, with the retinal ganglion cells being produced first and rods, bipolar, and Muller glial cells being produced last (Carter-Dawson and LaVail, 1979; Cepko et al., 1996a; LaVail et al., 1991; Stiemke and Hollyfield, 1995; Young, 1985). In contrast to the developing Drosophila retina, where precursor cells differentiate directly into neurons, the neurons of the vertebrate retina are generated from a pool of actively cycling neural progenitor cells. Neurogenesis in the vertebrate retina is characterized by several features. First, retinal progenitors are multipotent, and they can generate more than one or two cell types (Holt et al., 1988; Turner and Cepko, 1987; Turner et al., 1990; Wetts and Fraser, 1988). Second, despite the conserved birth order, there is an overlap in the generation of different retinal cell types (LaVail et al., 1991; Stiemke and Hollyfield, 1995; Young, 1985; see Chapter 25).

Vertebrate retinal progenitor cells are only able to give rise to certain subsets of cell types at different stages of development (Austin et al., 1995; Belliveau and Cepko, 1999; Belliveau et al., 2000). It has been shown that

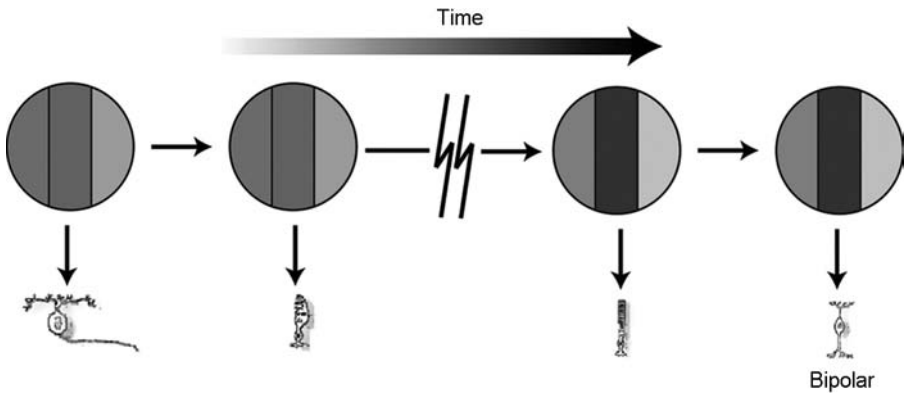

FIGURE 23.3 **The competence model for the temporal control of retinal neurogenesis.** Progenitors are proposed to undergo changes in competence or in their ability to give rise to particular cell types during the course of development. Evidence exists for both an early progenitor competence state and a late state in the vertebrate retina, as reflected by the different shading used. There is currently no evidence for the existence of an intermediate competence state. As discussed in the chapter, a temporal order of transcription factor expression has been shown to control the temporal order of neurogenesis in Drosophila.

although extrinsic signals can regulate the proportion of different cell types being made, they cannot alter the range of cell types generated. In combination with the multipotency of retinal progenitor cells, these observations led to the proposal of a competence model for retinal development (Figure 23.3), which suggested that retinal progenitor cells pass through a series of intrinsically determined configurations (or competence states). In each of these states, they are able to give rise to only a subset of cell types in response to extracellular signals (Cepko et al., 1996a).

Potentially, competence states might be determined by chromatin modulation, transcriptional states, gene expression profiles, translational regulation, protein accumulation/degradation, and posttranslational protein modification (Livesey and Cepko, 2001). There is some evidence for the transcriptional or translational control of progenitor competence. Two markers have been identified that show heterogeneity of expression in retinal progenitors: *syntaxin-1a* and *VC1.1* (Alexiades and Cepko, 1997). Retinal progenitors also display a changing response to mitogens over time, and the level of epidermal growth factor receptor expression is known to change over time in the retina (Lillien, 1995; Lillien and Cepko, 1992). In addition, the cyclin kinase inhibitors p27 and p57, which regulate cell cycle exit, are expressed in different subsets of progenitors (Dyer and Cepko, 2000b). It has also been suggested that the level of $p27^{xic1}$, which is another cyclin kinase inhibitor, increases over time in retinal progenitors and that its accumulation over a certain level is responsible for driving the formation of the final retinal cell type, the Muller glial cell (Ohnuma et al., 1999).

Two transcription factors, Prox1 and FoxN4, have been found to be necessary for the generation of overlapping classes of early-born cell types in the retina (Dyer et al., 2003; Li et al., 2004). Prox1 acts to both drive progenitor cells out of the cell cycle and to confer specific cell fates in the retina, most notably horizontal cell genesis (Dyer et al., 2003). FoxN4 is transiently expressed in a subset of retinal progenitor cells, and it is required for amacrine and horizontal cell genesis and differentiation (Li et al., 2004). However, where and when FoxN4 is required for the generation of those cell types are not clear.

The mechanisms that govern the switch between these different progenitor states or competences are unknown. It is possible that intrinsic factors, extrinsic factors, or a combination of the two are responsible for changes in progenitor competence. Several types of retinal neurons are known to produce signals that negatively feedback on the retinal progenitor cells, thereby regulating the types of neurons that they can generate (Bermingham et al., 1999; Reh and Tully, 1986; Waid and McLoon, 1998). A complicating factor in the understanding of retinal progenitor competence is the existence of heterogenous progenitor states at any one time (Brown et al., 1998; Dyer and Cepko, 2000a; Jasoni and Reh, 1996; Levine et al., 2000; Lillien and Cepko, 1992). This progenitor heterogeneity raises the complicating possibility that functionally different subsets of retinal progenitors exist and that each subset of progenitors may generate only a selection of retinal cell types.

D. A Temporal Identity for Neural Progenitor Cells and Their Progeny

In the asymmetrically dividing neuroblasts of the Drosophila embryo, a set of sequentially expressed transcription factors encoded by *hunchback, kruppel, castor, pdm,* and *grainyhead* has been identified (Brody and Odenwald, 2000; Isshiki et al., 2001). Although the expression of these factors occurs as a temporal sequence in neuroblasts, the daughter GMCs that they give rise to maintain the expression of the TF expressed in the mother neuroblast at the time that they were born. Both *hunchback* and *kruppel* have been shown to confer birth-order specificity on many neuroblast lineages, regardless of whether these lineages result in a neuronal or glial cell fate. How is this cascade of transcription factors regulated? Misexpression studies indicate that *hunchback* activates the expression of *kruppel* and that *kruppel* activates the expression of *castor* (Isshiki et al., 2001). Also, both *hunchback* and *kruppel* are known to repress the expression of the next plus one gene in the sequence: *hunchback* represses *castor,* and *kruppel* represses *pdm.* Interestingly, the overexpression of *hunchback* has been shown to reset the sequential expression of these transcription factors (Pearson and Doe, 2003). Recently, *seven-up* has been shown to be important for controlling the temporal switch from *hunchback* expression to *kruppel* in Drosophila neuroblasts. In *seven-up* mutants, an increase in early *hunchback*-positive neurons is observed, whereas the misexpression of *seven-up* results in a loss of these early-born neurons (Kanai et al., 2005).

The expression of this transcriptional cascade in many different neuroblast lineages (some of which give rise to neurons and some of which give rise to glia) suggests that it is responsible for conferring a temporal rather than an absolute identity on each GMC as it is born. As of yet, no such transcriptional cascade has been identified in vertebrates, although, given the similarity of the other steps of neural cell fate determination between flies and vertebrates, the existence of similar mechanisms would not be a surprise.

A role is emerging for a novel group of regulatory genes—the microRNAs—in this process. The nematode worm *Caenorhabditis elegans* homologue of *hunchback, hbl-1,* has recently been shown to control developmental time and to be regulated by the microRNA *let7* (Abrahante et al., 2003; Lin et al., 2003). Regulatory sites exist in the Drosophila *hunchback* 3′-untranslated region for the homologous Drosophila microRNAs, and it is therefore likely that it too is temporally regulated in this way. Regulation by microRNA genes

may therefore offer a novel mechanism for the temporal control of neurogenesis in conjunction with temporal TF sequences such as those described previously.

E. Cell Fate Determination Within a Given Competence State

Within an intrinsically defined progenitor competence state, cell fate has been shown to be influenced by extrinsic factors (e.g., by feedback inhibition from postmitotic neurons; Belliveau and Cepko, 1999; Reh and Tully, 1986; Waid and McLoon, 1998). Such a feedback mechanism has been shown to act on progenitor cells before M phase to affect daughter cell fate (Belliveau and Cepko, 1999), and a similar mechanism has been proposed for the developing neocortex (Desai and McConnell, 2000). It is noteworthy that extrinsic factors can also act to determine or respecify the fate of postmitotic cells, at least *in vitro*. For example, it is known that ciliary neurotrophic factor and leukemia inhibitory factor can cause cells that are destined to become rods to adopt aspects of the bipolar cell phenotype (Ezzeddine et al., 1997).

Notch signaling is known to be involved in the differentiation of the neurons and glia of the vertebrate retina and the developing forebrain. However, it is unclear whether Notch signaling has a permissive or instructive role in these processes (Livesey and Cepko, 2001). In the neural crest, transient Notch signaling is instructive in switching neural crest progenitors to neurogenesis and then to gliogenesis (Morrison et al., 2000). In the late retina, Notch acts to signal the transition between neurogenesis and gliogenesis (Furukawa et al., 2000), which it also does in the developing forebrain (Gaiano et al., 2000). Therefore, Notch signaling is likely to be important for regulating cell fate determination in vertebrates.

IV. COMMON FEATURES OF CELL FATE DETERMINATION IN DIFFERENT REGIONS OF THE NERVOUS SYSTEM

In other regions of the developing vertebrate nervous system, cell fate determination is similar to the situation seen in the retina. The progenitor cells of both the cortex and the spinal cord are multipotent (Briscoe et al., 1999; Leber et al., 1990). In the developing cortex (as in the retina), progenitors give rise to neurons before generating glial cell types (Morrison et al., 2000; Qian et al., 2000). Cortical progenitors progress through phases that are reminiscent of the competent states of retinal progenitors in which they are competent to produce cells of a given laminar fate (Morrison et al., 2000; Qian et al., 2000). However, unlike the situation in the retina, cortical neural progenitor cells are capable of generating later (but not earlier) cell types after heterochronic transplantation (Desai and McConnell, 2000; McConnell, 1988). This has led to the concept of the progressive restriction model in cortical cell fate determination (Desai and McConnell, 2000).

Both cortical and spinal cord progenitors can respond to extrinsic factors that regulate their cell fate choices. Cortical progenitors are competent to respond to extrinsic signals until late/early G2 in the cell cycle (McConnell, 1988), and this is in agreement with the finding that retinal progenitors make cell fate choices before M phase (Belliveau and Cepko, 1999). As in the retina, feedback signaling is used as a mechanism of neural cell fate determination in

the spinal cord, where postmitotic motor neurons induce the genesis of interneurons (Pfaff et al., 1996). Interestingly, cytokinesis has been shown to be essential in Drosophila for the temporal switching of neuroblast TF expression and the temporal order of neurogenesis, which suggests that feedback from newly generated daughter cells to stem/progenitor cells may be a feature of the general mechanism for driving neural stem and progenitor cells between competence states (Grosskortenhaus et al., 2005). Heterogeneity also appears to be a conserved feature of vertebrate neurogenesis, because different populations of spinal cord progenitors can be distinguished by the expression of different transcription factors (Briscoe et al., 2000).

V. PERSPECTIVES

Although many advances have been made in the understanding of neural cell fate determination, many questions remain. For example, the transcriptional networks underlying neural progenitor cell identity and competence remain to be fully elucidated. In the future, the use of genomics technologies, such as global gene expression profiling and TF binding site analysis, will allow such questions to be addressed in combination with functional approaches.

An understanding of neural cell fate determination is important for the understanding and treatment of neural disease and injury in humans. In the future, a molecular understanding of neural cell fate determination may be important for the development of therapies that can repair damage to the nervous system by stimulating endogenous neural stem cells. Additionally, such an understanding may allow for the development of *in vitro* strategies to generate specific neurons from embryonic or neural stem cells for use in transplantation therapies to replace lost or damaged neurons. Finally, an understanding of the cell and molecular biology of neural cell fate determination will be essential to the development of diagnostics and therapies for a range of neurodevelopmental disorders, including autistic spectrum disorders.

SUMMARY

- Proneural genes encode TFs that are responsible for initiating the development of neuronal lineages in both vertebrates and mammals by promoting the generation of neural progenitor cells.
- Neuronal differentiation genes encode TFs that function downstream of proneural genes and that are responsible for neural cell fate determination and differentiation.
- Neural progenitor cells acquire a distinct spatial identity first through the division of the nascent CNS into discrete territories and regions and then through the spatial patterning of individual regions.
- Within each region, neural progenitor cells are multipotent and give rise to different types of neurons over time through the process of asymmetric division.
- The competence of neural progenitor cells to generate different neuronal progeny changes over time as a result of the action of both intrinsic and extrinsic mechanisms.

GLOSSARY

Cell fate
The cell type that a cell will become. This term does not imply commitment or differentiation, only that the cell will eventually become a certain type.

Commitment
An irreversible decision to produce or become a particular cell type. This is defined operationally as the refusal of a cell to change its fate when exposed to various different environments.

Competence
The ability of a cell to respond to a cue or a set of cues to produce a defined outcome.

Differentiation
The elaboration of particular characteristics expressed by an end-stage cell type or by a cell that is en route to becoming an end-stage cell. This term is not synonymous with commitment, but differentiation features are used to determine when a cell is committed.

Multipotent
The ability of a stem or progenitor cell to generate progeny of more than one fate. Lineage analysis has demonstrated that retinal progenitor cells are multipotent. However, other experiments have shown that the cells are limited in their competence to make particular cell types at particular times. Thus, competence is a temporally defined ability that does not show the overall potency of a cell; for example, early retinal progenitor cells cannot respond to late environments by producing late cell types within 1 to 2 days, although their daughters will eventually become late cell types.

Progenitor cell
A mitotic cell that is not capable of indefinite self-renewal and that will produce a limited repertoire of cell types.

Proneural gene
A basic helix–loop–helix transcription factor that is both necessary and sufficient to initiate the development of neuronal lineages and to promote the generation of progenitor cells that are committed to neuronal differentiation.

Specification
The process by which a cell that is competent to make a particular cell type is directed along the pathway to become that cell type. Such a cell might not be committed to that fate, in which case its differentiation can be reversed and another fate can be achieved through respecification.

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RECOMMENDED RESOURCES

GenePaint: www.genepaint.org—A database of section *in situ* hybridization images of the developing mouse brain from Gregor Eichele's group.
Guillemot F.: Cellular and molecular control of neurogenesis in the mammalian telencephalon, *Curr Op Cell Biol* 17:639–647, 2005.

Livesey FJ, Cepko CL: Vertebrate neural cell-fate determination: lessons from the retina, *Nat Rev Neurosci* 2:109–118, 2001.

VisiGene: http://genome.ucsc.edu/cgi-bin/hgVisiGene—Part of the University of California Santa Cruz genome browser Web site, an excellent resource for developmental gene expression in the mouse, which collates many public in situ hybridization databases.

24 PATHFINDING AND PATTERNING OF AXONAL CONNECTIONS

STEPHANIE A. LINN, STEPHANIE R. KADISON, and CATHERINE E. KRULL

University of Michigan, Department of Cell and Developmental Biology, Ann Arbor, MI

INTRODUCTION

After their induction, neurons differentiate in part by extending their axons to innervate particular targets and by growing dendrites. Each neuron must be connected precisely to neighboring cells to drive functional behaviors, including locomotion and sensory perception. How do neurons find their way to their targets? The idea is that neuronal cell bodies extend their growing axon shafts, which are tipped by growth cones or sensors at their distal ends. These growth cones sense cues in the extracellular environment and respond to them by growing toward or away from these cues. Axonal growth cones are guided along specific routes by attractive and repulsive cues in the extracellular environment (Figure 24.1). Attractive cues are thought to guide axons by influencing them positively, providing a substrate on which axons can crawl easily, or permitting axons to remain on a certain track. By contrast, repulsive cues keep axons out of certain territories. The combination of attractive and repulsive cues is likely crucial to the formation of precise neural circuitry (Table 24.1).

Attractive and repulsive cues that guide axons can act at short range or long range (see Figure 24.1). Short-range cues that guide axons typically involve cell–cell or cell–substrate contact, whereas long-range cues are often synthesized and secreted at a distance from the growing axon and act in a morphogen gradient to guide axons.

Genetic and biochemical studies have led to the identification of several families of guidance molecules, including netrins, Slits, semaphorins, and ephrins (Figure 24.2). In addition, cell adhesion molecules (CAMs) and extracellular matrix (ECM) molecules are known to guide growing axons. These guidance molecules must impinge on the growth cone of the neuron to exert their effects. Guidance molecules direct axons by regulating the cytoskeletal components of the growth cone by activating certain signaling pathways. However, this process remains poorly understood.

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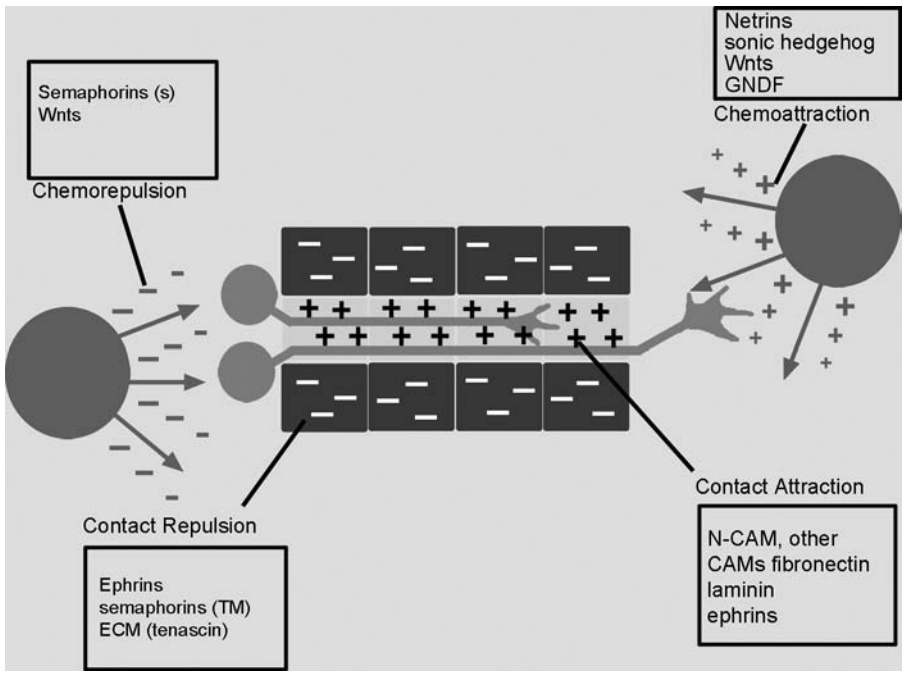

FIGURE 24.1 Molecules influence axon pathfinding and patterning. Some of these molecules can act as morphogens and serve as chemoattractants (+) or chemorepellents (−). In these cases, these molecules would act over distances to influence cell behavior. In other examples, some of these molecules require cell contact and mediate contact attraction (+) or contact repulsion (−). These kinds of molecules are often located on cells or near cells and signal locally. Examples of the types of molecules that are chemorepellents, chemoattractants, contact repellents, or contact attractants are located in the boxes in this figure. *TM,* Transmembrane proteins; *s,* secreted proteins.

TABLE 24.1 What are the Criteria for a Molecule to be Required in Axon Guidance?

1. The growth cone must have receptors for the ligand. Alternatively, the growth cone must synthesize and secrete or insert the ligand on its cell membrane.
2. The ligand or receptor must be expressed locally on the pathway or at a distance, near the final destination of the axon.
3. The blocking of receptors and/or ligands must result in axon-guidance defects.
4. The signaling pathways downstream of a receptor or ligand must be activated in the growth cone to establish a response to the ligand or receptor, respectively.

In this chapter, we will examine the guidance molecules that establish axonal connectivity. First, we will examine the roles of transcription factors in developing neurons and in the promotion of axon guidance. Then, the influence of guidance cues in two well-known systems (at the midline and in the periphery of the developing limb) will be described. We will examine known roles of CAMs and activity in axon guidance. In addition, we will use the retinotectal or retinocollicular system as a model for studies of axonal connectivity. Finally, the underlying cell biology of axon guidance will be discussed.

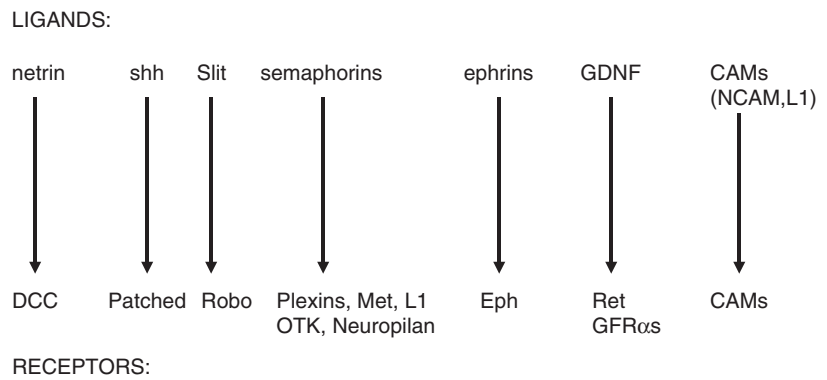

FIGURE 24.2 **Signaling during axon pathfinding and patterning involves several secreted or cell-attached ligands and their receptors.** Various ligands are listed that are known to be involved in axon pathfinding/patterning. Their corresponding receptors, shown at the ends of the arrows, are localized to the plasma membrane of developing neurons.

I. TRANSCRIPTION FACTORS IN CELL SPECIFICATION AND AXON CONNECTIVITY

Several transcription factors are expressed in developing neurons and are thought to play a central role in the establishment of axon connectivity. In general, these transcription factors are thought to confer cell fate on neurons, instructing them, for example, to become motor neurons, interneurons, or commissural neurons. However, recent studies suggest that transcription factors are involved directly and indirectly in guiding growing axons to their final targets.

Various transcription factors appear to function in cell specification in the developing spinal cord, including the LIM family (Figure 24.3; Jessell, 2000). As an example, motor neurons are born in the ventricular zone and migrate laterally to take residence in the lateral motor column (LMC) in the chick and mouse at limb levels. The expression of LIM transcription factors defines the subdivision of the LMC into lateral and medial cohorts of motor neurons. Early during development, all neurons that will form the LMC express the transcription factor Olig2 and LIM family member Islet1. Later, Olig 2 expression is extinguished, but Islet1 expression is maintained only in LMC(m) motor neurons; it is eliminated in postmitotic LMC(l) neurons that begin to express an alternative LIM transcription factor, Lim1.

When neural tube cells are transplanted to new anterior–posterior positions, the expressions of the LIM family of transcription factors and motor neuron identity are altered in concert, which suggests that these factors control motor neuron fate specification in the LMC (Matise and Lance-Jones, 1996). Mice lacking Islet1 generate few motor neurons, which indicates that Islet1 is required for motor neurons to develop. Moreover, double knockout mice in which Lhx3 and Lhx4 are absent demonstrate that these LIM gene products are required for the differentiation of a specific subset of motor neurons (Sharma et al., 1998). However, the potential roles of LIM family transcription factors in later events of axon pathfinding have been difficult to explore. Most LIM gene family mutants are lethal to embryos, which makes it hard to uncover their roles in axon connectivity. However, recent

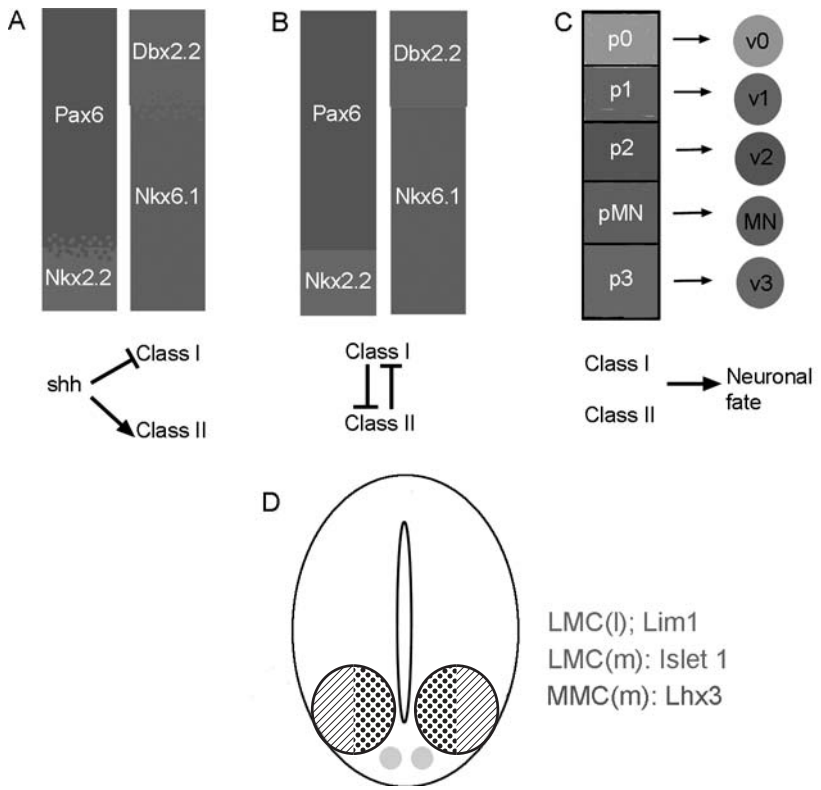

FIGURE 24.3 **Transcription factors act to pattern cell types in the neural tube.** The three-step model of ventral neural pattering is shown. Boxed areas represent areas of the neural tube, with dorsal at the top and ventral at the bottom. **A,** During the early stages, the secreted protein sonic hedgehog *(Shh)* represses class I genes *(Pax6, Dbx2)* but activates class II genes *(Nkx2.2, Nkx6.1)*. **B,** Class I genes repress class II genes and vice versa, providing sharp borders between progenitor domains. **C,** Other transcription factors (i.e., *Olig2*) act to promote the development of interneurons *(v0–3)* or motor neurons *(MNs)*. **D,** Schematic diagram showing the neural tube with neurons organized into columns during later stages of development. At the limb levels, motor neurons lie in the lateral motor column *(LMC)*. The LMC is further subdivided into the lateral part of the LMC (*LMC(l); striped*) and the medial part of the LMC (*LMC(m); spotted*). Motor neurons in the LMC(l) express the transcription factor Lim1, whereas motor neurons in the LMC(m) express the transcription factor Islet1. Motor neurons expressing Lim1 project their axons to the dorsal limb, whereas motor neurons expressing Islet1 extend axons to the ventral limb. Motor neurons in the medial part of the medial motor column (*MMC(m)*) express Lhx3 protein *(light gray)* and project to body wall muscle.

studies have shown that a lack of Lim1 function randomizes axon projections from LMC(l) neurons into the dorsal and ventral limb (Kania et al., 2000). Furthermore, the ectopic expression of Lim1 in cells that normally do not express it also regulates the expression of EphA4, a receptor tyrosine kinase (RTK) involved in axon pathfinding by motor neurons in the neural tube that links cell specification and axon pathway selection (Kania and Jessell, 2003).

The organization of young neurons into columns is a prominent feature of the central nervous system (CNS). In many regions of the CNS, the grouping of neurons into columns connects cell body position with their axonal

trajectory, thus leading to the establishment of precise topographic maps. The organization of the CNS into columns has best been studied in the spinal cord, where distinct subsets of motor neurons innervate distinct targets in the periphery. All motor neurons come from progenitor cells that are located at the same dorsoventral position of the developing spinal cord. However, motor neurons seem to achieve their unique columnar identities as a function of their position along the rostrocaudal axis of the spinal cord (Ensini et al., 1998; Lance-Jones et al., 2001; Shah et al., 2004).

Signals from the paraxial mesoderm influence the columnar fate of motor neurons. When the paraxial mesoderm is transplanted between the limb and thoracic levels, the LMC is respecified (Ensini et al., 1998). Signals from the node and notochord (axial mesodermal tissues) also regulate the identity of motor neurons (Omelchenko and Lance-Jones, 2003). In particular, members of the Hox family are expressed by motor neurons at distinct rostrocaudal positions, and their expression changes along with cell identity when brachial and thoracic neural tubes are transposed. Thus, Hox genes and their protein products are excellent candidates for the early assignment of motor neuron identity (see Chapter 9).

Members of the Hox-c cluster of proteins localize in particular to motor neurons in the spinal cord. Hoxc6 expression by motor neurons is confined to brachial levels, whereas Hoxc9 expression marks motor neurons at thoracic levels. Using LIM family transcription factors to determine which subsets of motor neurons in columns were labeled, Hoxc6 and Hoxc9 expression was found to be coincident with brachial LMC and thoracic columns of motor neurons, respectively. To determine if changes in Hox proteins lead to alterations in motor neuron fate, Hoxc9 was ectopically expressed at brachial levels of the spinal cord, or Hoxc6 was expressed at thoracic levels. This led to corresponding changes in motor neuron identity at these different axial levels, supporting the idea that Hoxc genes confer brachial and thoracic identity to motor neurons in the spinal cord (Dasen et al., 2003). Moreover, findings indicate that Hox proteins confer the selectivity with which spinal motor neurons innervate target muscles in the developing forelimb by defining motor neuron subtypes (Dasen et al., 2005). Together, these findings support a strong role for a genetic regulatory network that includes Hox genes in the establishment of motor neuron identity and connectivity with target muscles.

One transcription factor alone has been shown to act directly as a guidance factor for growing axons: Engrailed-2 (En-2). En-2 is a homeodomain transcription factor that is expressed in a caudal-to-rostral gradient in the developing midbrain, where it patterns the optic tectum. Surprisingly, En-2 also repels or attracts growing Xenopus axons specifically in a growth cone turning assay (Brunet et al., 2005). Furthermore, En-2 protein can accumulate inside growth cones, whereas a mutant form of En-2 that cannot enter cells does not accumulate and does not cause axons to turn. When inhibitors of protein synthesis are applied, the turning responses to En-2 are abolished. Finally, the phosphorylation of translational regulatory proteins is enhanced when En-2 is added to growth cones. Together, these results and others strongly indicate that the transcription factor En-2 is a secreted protein that influences axon guidance and that it may help to organize retinotectal/retinocollicular projections in addition to regulating the expression of ephrin-As in the tectum (Friedman and O'Leary, 1996).

II. AXON PATHFINDING AT THE MIDLINE

The ventral midline of the CNS is a commonly employed model system for axon pathfinding and the patterning of axonal connections in vertebrate and invertebrate systems (Figure 24.4). In vertebrates, commissural axons (CAs) extend circumferentially toward the floor plate, which is an intermediate target located at the ventral midline of the spinal cord (Kaprielian et al., 2001). After traversing the floor plate, most (but not all) CAs abruptly alter their direction of growth from the transverse to the longitudinal plane (Kadison and Kaprielian, 2004). The fact that the transition from transverse to longitudinal growth occurs at the floor plate implies that this intermediate target may be at least partially responsible for providing the cues necessary to implement this behavioral switch.

The importance of floor-plate–derived guidance cues has been highlighted in various studies of floor-plate–lacking mutants. In previous analyses of mouse and zebrafish lacking either a floor plate or both a floor plate and a notochord, CAs were observed to extend wild-type–like trajectories to the ventral midline, but these trajectories exhibited pathfinding errors after they arrived there (Bovolenta and Dodd, 1991; Matise et al., 1999). In floor-plate–lacking *gli2*-deficient mice, for example, many CAs were reported to cluster at the midline. In a zebrafish floor-plate–lacking mutant, many CAs displayed wild-type–like projection patterns along the longitudinal axis, which suggests that the floor plate may not be absolutely required for midline crossing and/or the maintenance of decussated projections (Bernhardt et al., 1992; Hatta, 1992). Interestingly, a recent study reported that, in the absence of a floor plate, a number of decussated CAs were capable of following wild-type–like trajectories in the embryonic mouse spinal cord at older ages both *in vivo* and *in vitro* (Kadison et al., 2006a).

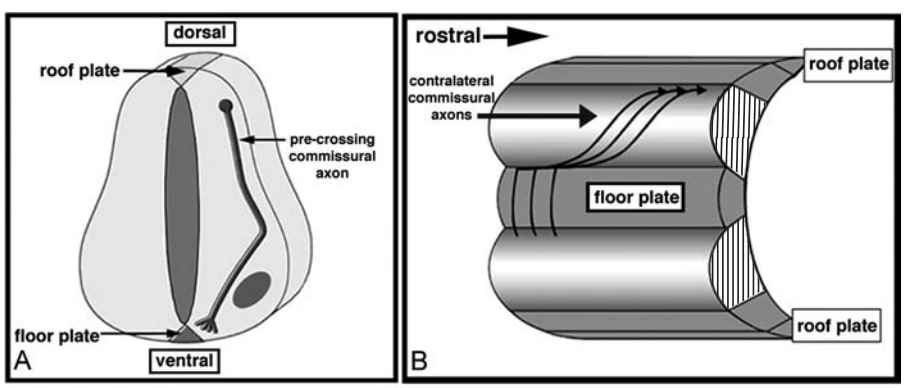

FIGURE 24.4 **Commissural axons must navigate across the midline. A,** In vertebrate animals, commissural neurons are located in the dorsal neural tube, and they send their axons ventrally, toward the floor plate. **B,** In an open book explant in which the developing neural tube is bisected at the roof plate and flattened, commissural axons have crossed the floor plate, and they extend in the contralateral neural tube *(striped)*.

A. Reaching the Ventral Midline

Much is known about the mechanisms that regulate the pathfinding of CAs to the ventral midline. A dorsal midline structure known as the roof plate has been shown to reorient precrossing CAs *in vitro*, which suggests that guidance cues contained within this structure may be responsible for the extension of these axons away from the dorsal midline. Three candidates—BMP7, BMP6, and GDF7—that are expressed in the roof plate were tested in an *in vitro* reorienting assay, but only BMP7-expressing cells were able to mimic the level of repulsion formerly shown to be associated with roof plate cells (Augsburger et al., 1999). Interestingly, when GDF7 was added in conjunction with BMP7 *in vitro*, the repulsion was augmented. In a separate biochemical assay, it was shown that BMP7 and GDF7 are capable of forming heterodimers, which suggests that they may function as such *in vivo*. Consistent with these *in vitro* findings, CAs in $BMP7^{-/-}$, $GDF7^{-/-}$, and $BMP7^{-/-}/GDF7^{-/-}$ embryos made pathfinding errors, including extending axons medially and crossing the dorsal midline. The fact that GDF7 alone did not mimic roof plate repulsion *in vitro* but that it was shown to be important for propelling the dorsal commissural axon away from the dorsal midline *in vivo* suggests that GDF7 does not inherently contain repulsive activities for CAs but rather that it must work in concert with BMP7, possibly as a heterodimer or through some other mechanism that has yet to be determined (Augsburger et al., 1999; Butler and Dodd, 2003). One of the major effects of this study is that a classic morphogen, BMP7, was shown to also act as a guidance cue. BMP7 is primarily known for its role in the specification of dorsal spinal interneurons, and this occurs only 1 day before its role in commissural axon guidance.

Complementary to the way in which roof-plate–derived cues propel CAs away from the dorsal midline, cues derived from the floor plate draw CAs toward the ventral midline. Almost 20 years ago, a diffusible chemotropic factor for CAs was found to emanate from floor plate cells *in vitro* (Tessier-Lavigne et al., 1988). It was later determined that this secreted cue also has the ability to reorient CAs in culture and that it is possibly the most well-known guidance cue, netrin-1 (Kennedy et al., 1994). An *in vivo* role for netrin was later established in the analysis of mice with a null mutation of the netrin receptor Deleted in Colorectal Cancer. An analysis of these mutants revealed a phenotype with which many CAs were unable to navigate toward or across the floor plate. Unexpectedly, floor plate tissue derived from netrin-1 hypomorphic mice was still capable of reorienting CAs *in vitro*, thus demonstrating that netrin is not the sole floor-plate–derived chemoattractant (Serafini et al., 1996). On the basis of these results, a recent study was carried out with the aim of identifying other putative chemoattractants derived from the floor plate (in addition to netrin). To do this, the authors crossed the gli2, floor-plate–deficient mouse with the netrin-1 hypomorphic mouse. These double mutants displayed a more dramatic phenotype than the netrin mutant; almost all CAs were foreshortened or projected medially rather than toward the ventral midline. As a next step, the authors chose a candidate molecule expressed in the floor plate, Sonic hedgehog (Shh), which is known primarily to act as a morphogen during early development. They showed that Shh can reorient CAs and that the blockade of Shh signaling reverses this activity *in vitro*. In addition, they observed a high level of defasciculation and fewer

CAs reaching the floor plate in a mutant mouse lacking Smoothened (Smo), a canonical signaling mediator of Shh (Charron et al., 2003).

B. Crossing the Floor Plate

After CAs arrive at the ventral midline, they must then successfully traverse the floor plate to reach the contralateral side of the spinal cord. It has been shown in the developing chick spinal cord that the CAMs belonging to the immunoglobulin (Ig) superfamily, Nr-CAM and axonin-1, are required for passage across the floor plate. Nr-CAM is localized to floor plate cells, whereas axonin-1 is expressed on CAs. In experiments perturbing Nr-CAM or axonin-1 *in ovo* using either function-blocking antibodies or RNAi, there was a marked decrease in the number of axons that were able to cross the floor plate (Stoeckli and Landmesser, 1995; Pekarik et al., 2003). In a separate but related set of studies, CAs were unable to enter floor-plate explants when function-blocking antibodies against Nr-CAM/axonin-1 were added to the cultures, thereby supporting the idea that Nr-CAM relieves the inhibition that CAs may perceive from floor-plate cells as they decussate (Stoeckli et al., 1997).

Another family of guidance cues involved in midline crossing in both vertebrates and invertebrates includes the Slit/Robo family. Slit, which is a large ECM protein, was first shown to act as a midline-associated repellent based on the phenotype observed in the Slit mutant in flies. In these mutants, CAs projected within the ventral midline as a single longitudinal fascicle rather than forming their usual ladder-like configuration in the ventral nerve cord (Rothberg et al., 1990). Interestingly, it appears that the repulsive functional role of Slit at the midline has been evolutionarily conserved from flies to higher vertebrates (Brose et al., 1999). Recently, it was shown that CAs in Slit1–3 triple knockout mice stall at the ventral midline and even occasionally recross the floor plate. Similar midline defects were also observed in the chick when reagents interfering with Slit/Robo binding were applied *in ovo*. Robo, which is the receptor for Slit, is expressed selectively on longitudinal axons in flies, and the loss of Robo results in multiple recrossing events at the ventral midline of the nerve cord (Seeger et al., 1993).

The interpretation of this phenotype and its relationship to the Slit mutant phenotype was not immediately clear. It was not until the discovery of comm that the relationship between Slit and Robo was uncovered. In wild-type flies, comm is expressed in midline glia and on CAs only as they are decussating. In the comm fly mutant, a complete absence of commissures was observed in the ventral nerve cord, which gave rise to its name, commissureless (Tear et al., 1996). The interplay between Robo, Slit, and Comm required to regulate midline crossing in flies was later shown to depend on both the spatial and temporal precision of gene/protein expression. Through various mutant analyses, it was established that comm is responsible for regulating the segment-specific expression pattern of Robo, and it does so by transferring Robo into endosomes before and during midline crossing. After CAs have decussated, Comm presents Robo on the surface of the growth cone in a cell-autonomous manner through an as yet unidentified mechanism (Keleman et al., 2005). In summary, the strict regulation of Robo expression via comm ensures that CAs are insensitive to midline-associated Slit until after decussating, thereby facilitating midline crossing in flies.

In the mouse, Rig-1 (Robo3) homozygous mutants phenocopy the comm mutant, although they are molecularly distinct cues. Rig-1 protein has been shown to be selectively expressed on precrossing CAs in the mouse and chick (Sabatier et al., 2004), and, in Rig-1 homozygous mice, there is a complete absence of commissures at the ventral midline at all anterior–posterior levels of the CNS (Marillat et al., 2004). Although the mechanism through which this occurs is still not entirely clear, *in vitro* evidence suggests that Rig-1 negatively modulates Slit sensitivity. It was proposed that, in the absence of Rig-1, CAs are unable to overcome the repulsion of midline-associated Slit and, as a result, never cross the floor plate in Rig-1 homozygous mice. Recently, floor-plate–associated ephrin-B3 and its cognate receptors were shown to regulate the frequency of decussation of a specific commissural axon subtype in the mouse, potentially conferring another level of regulation to guidance molecules (Kadison et al., 2006b).

C. Leaving the Midline

After CAs cross through the floor plate, they must then leave the midline to navigate toward their next choice point. For CAs to do this, they must first lose their responsiveness to floor-plate–derived chemoattractants. This is precisely what has been shown to occur for decussated CAs, at least in the hindbrain. CAs that have previously crossed through a floor plate were shown to be insensitive to an ectopically positioned floor plate or to netrin-expressing cells *in vitro* (Shirasaki et al., 1998). The potential mechanism underlying this loss of netrin attraction was delineated in a series of elegant experiments involving the Xenopus turning assay. At early stages of commissural axon growth, isolated Xenopus spinal axons were exposed to either netrin or Slit proteins. In this scenario, the axons were insensitive to Slit but turned toward the source of netrin, thereby reflecting their selective responsiveness to this attractant. At a later stage, however, the profile of responsivity drastically changed: the spinal axons were now insensitive to netrin but repelled by Slit. When younger spinal axons were exposed to both Slit and netrin concurrently, they failed to elicit any response, leading the authors to investigate the mechanism underlying this apparent silencing of netrin attraction. Through a series of biochemical assays and elegant chimera studies, they showed that netrin silencing is the result of a direct interaction between the cytoplasmic domains of Deleted in Colorectal Cancer and Robo (Stein and Tessier-Lavigne, 2001).

Along with this loss of attraction to midline-derived chemoattractants, CAs have also been shown to gain responsiveness to midline-derived repellents *in vitro*. An *in vitro* assay system with two separate configurations of spinal cord explants was used: one configuration consisted of a half spinal cord with a floor plate attached, and the other contained dorsal spinal cord tissue. This approach was used to assay the response of precrossing (dorsal spinal cord) and postcrossing (floor-plate–attached) CAs to various guidance cues. Of the molecules assayed, Semaphorin (Sema) 3B, Sema 3F, and Slit2 were the only cues capable of inhibiting the growth of postcrossing (but not precrossing) CAs *in vitro* (Zou et al., 2000). The authors proposed that these repellent guidance cues were instructive for expelling CAs out of the vicinity of the ventral midline and into longitudinal tracts. Defects consistent with this role were observed in mice lacking Neuropilin-2, a receptor expressed

on precrossing and postcrossing CAs that preferentially bind to class 3 Semas (Chen et al., 1997).

D. Longitudinal Axon Guidance

At early stages of commissural axon growth, nearly all decussated axons extend rostrally in the embryonic rodent spinal cord (Bovolenta and Dodd, 1990). A recent series of experiments have begun to uncover the mechanism underlying this decision to make an anterior turn. Using a candidate gene approach, a number of candidates were identified that belonged to the Wnt family, including Wnt4, which was expressed in a gradient along the anterior–posterior axis of the ventral midline. Functionally, the authors found that Wnt4 acted as an instructive attractive cue for CAs turning rostrally within spinal cord explants *in vitro*. These results were verified *in vivo* in an analysis of a mouse lacking the gene encoding Frizzled3, a receptor for Wnts. In this mutant, the authors observed many CAs displaying random anterior–posterior turning behavior. In the chick spinal cord, a second anterior–posterior cue was discovered through a series of *in ovo* RNAi experiments illustrating that Shh may act as a chemorepellent for decussated CAs. Shh mRNA is expressed in a gradient counter to the one observed for Wnt4 mRNA in the rodent ventral midline, which supports the idea that Shh may act as a repulsive cue to push CAs into the anterior direction. Although Smo was shown to be responsible for mediating the Shh-dependent attraction of CAs to the floor plate (Charron et al., 2003), cyclopamine (which blocks Smo-mediated Shh signaling) did not affect the anterior–posterior guidance of postcrossing CAs. By contrast, blocking the function of HIP (a protein that interacts with Shh) with RNAi recapitulated the anterior–posterior errors observed in the Shh perturbation experiments (Bourikas et al., 2005). It appears that one solution that CAs have devised for responding to attraction or repulsion from the same guidance cue includes using distinct receptors, at least in the case of Shh.

During the late stages of commissural axon growth in vertebrates, the majority of rostrally directed contralateral CAs extend into intermediate regions of the spinal cord (Imondi and Kaprielian, 2001; Kadison and Kaprielian, 2004). The nature of how CAs maintain their dorsoventral position is a largely unexplored question in the rodent. In the fly, it appears that the selective expression of different combinations of Robo receptors on decussated axons, which has been called the *Robo code,* assigns a dorsoventral position to longitudinally projecting axons (Rajagopalan et al., 2000; Simpson et al., 2000). Interestingly, it appears that this role of Robo has been phylogenetically conserved in the mouse.

E. Axon Pathfinding to the Limb

During neural development, spinal motor axons extend in a precise manner from the ventral portion of the developing spinal cord to innervate muscle targets in the limb. The stages of development when motor axons grow to innervate their limb targets have been defined by elegant studies from Landmesser et al. (1978) in the chick, and they will be described at hind-limb levels (Figure 24.5). Initially, outgrowing axons associate with one another, bundle together in the proximal spinal nerves, and reach the base of the hind limb at stage 21. These axons stall at the limb base until stage 23, when they defasciculate at the limb base and segregate into target-specific fascicles,

thereby entering the hind limb by a dorsal or ventral trajectory. At stage 28, the pattern of innervation of target muscles in the limb is complete and mature.

There are several major guidance cues that contribute to the growth and pathfinding of axons during their navigation to the limb. One of these sets of cues is the Eph family of RTKs and their ephrin ligands (see Chapter 22). The Eph family comprises the largest family of RTKs, and these are categorized into two subfamilies, A and B. EphA RTKs bind the glycophosphatidylinositol-anchored ephrin-A ligands, and EphB RTKs prefer the transmembrane ephrin-B ligands (Gale et al., 1996), although recent studies suggest that binding between Ephs and ephrins is more promiscuous across subclasses (Himanen et al., 2004). Typically, interactions between Ephs and ephrins require cell contact and are involved in repulsive interactions. Cells expressing Eph receptors avoid territories that express ephrins. However, in certain cases, members of the Eph family can promote positive contacts between cells (Eberhart et al., 2004; Chen et al., 2004).

The expression of Eph family members is complicated during the time that motor axons grow to the limb and find their targets. This complicated expression pattern illustrates the importance of defining the localization of mRNAs and proteins during the entire process of axon pathfinding (Eberhart et al., 2000). In the developing spinal cord, EphA4 protein is initially broadly expressed by all motor neurons and their axons, which arrive at the limb base (stage 21) but then become segregated to the forming dorsal nerve trunk at the crural plexus, when motor axons start to sort and change their nearest neighbor relationships (stage 23). At stage 28, EphA4 remains strong on the dorsal nerve trunk. In addition, EphA4 protein is also present in the dorsal–proximal

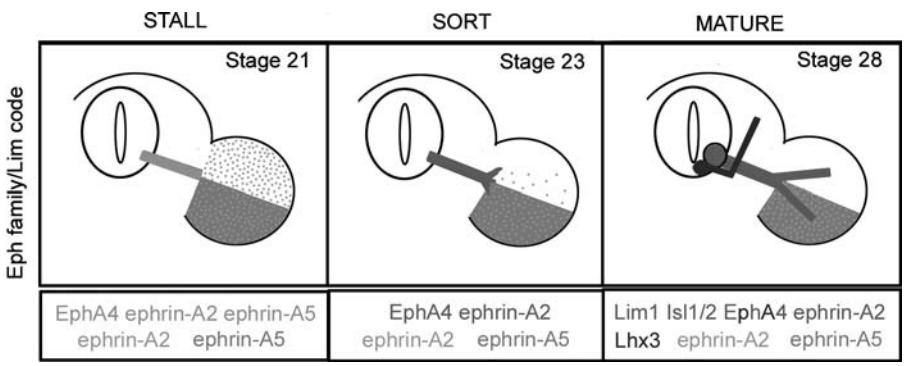

FIGURE 24.5 Motor axons that project to their target muscles in the limb show the dynamic expression of transcription factors and Eph family members. When motor axons stall at the base of the limb *(left panel)*, they express EphA4 protein and its ligands, ephrin-A2 and ephrin-A5. When motor axons sort *(middle panel)*, ephrin-A5 protein is no longer apparent on axons, and EphA4 becomes gradually segregated to the dorsal nerve trunk. When motor neurons have matured and innervated target muscles *(right panel)*, motor neurons in the LMC(l) express Lim1, EphA4, and ephrin-A2; motor neurons in the LMC(m) express Islet1 and ephrin-A2. Ephrin-A2 protein *(light blue)* is also expressed broadly across the limb mesoderm when the motor axons stall, and it becomes gradually expressed in the ventral limb. Ephrin-A5 protein *(dark blue)* localizes to the ventral limb throughout the stages of motor axon pathfinding. (See color insert.)

limb mesoderm from the earliest stages; its function here is unknown. Two ligands for EphA4, ephrin-A2 and ephrin-A5, are also found on all motor axons that project to the base of the limb. Ephrin-A5 protein is quickly downregulated on all motor axons, although its mRNA and protein remain localized to LMC motor neurons. Ephrin-A2 protein remains localized to all motor axons as they sort and enter the limb. Ephrin-A2 and -A5 proteins are also expressed in the limb mesoderm. When motor axons arrive at the limb base and stall, a swath of ephrin-A extends across the limb mesoderm. When motor axons start to sort, the expression of ephrin proteins in limb mesoderm starts to become restricted to the ventral half, and it remains so at mature stages (stage 28).

Studies have shown that EphA4 RTK is required and sufficient for motor axons to project dorsally in the limb (Helmbacher et al., 2000; Eberhart et al., 2002; Kania and Jessell, 2003). When EphA4 is deleted in the mouse, motor axons project randomly in the limb. When EphA4 is ectopically expressed in motor neurons that do not normally do so, EphA4-positive axons are driven dorsally in the limb. This is thought to happen because ephrin-A5 is expressed in the ventral limb mesoderm and avoided by EphA4-positive axons. However, another subset of motor neurons in the medial portion of the medial motor column (MMC(m)) expresses EphA4 and responds in a positive manner to ephrin-A5. Normally, these motor neurons send axons into the rostral half somite and innervate body wall muscle. When EphA4 signaling is blocked in MMC(m) neurons or when ephrin-A5 is ectopically expressed in the developing somites, axons of MMC(m) neurons grow aberrantly into caudal half somites. Here, ephrin-A5 has a negative or inhibitory effect on limb-innervating motor neurons but a positive effect on motor neurons that innervate body wall muscle. Together, these data support an important point: assumptions about the function of a particular receptor or ligand in axon pathfinding cannot be made globally for a set of cells that appear to be homogeneous. Instead, the function of a receptor or ligand must be tested in precise populations of neurons.

How are these differential effects on growing axons mediated? In this case, EphA4-positive motor neurons that innervate the limb express the downstream intracellular signaling molecule ephexin, which is a guanine nucleotide exchange factor (GEF); motor neurons that innervate body wall muscle do not (Sahin et al., 2005). When ephexin is specifically reduced in LMC neurons using a shRNA approach, there is a striking effect on motor axons: they enter the limb prematurely as compared with the control side, where motor axons are still stalled. Together, these findings reinforce the notion that there are different signaling molecules and cascades downstream of EphA4 in different subsets of neurons. Additionally, these results indicate that ephexin is required for motor axons to stall at the base of the limb.

F. Semaphorins and Their Receptors as Axon Pathfinding Cues

Not all guidance cues are cell-contact dependent. Some cues are capable of acting over short or long distances; the semaphorins are an example of this mode of signaling. One of the largest families of growth-cone–guidance proteins, semaphorins are categorized into eight classes based on sequence and structure: classes 1 and 2 are found in invertebrates; classes 2 through 7 are found in vertebrates; and class V is made up of viral semaphorins. Semaphorins can be secreted glycophosphatidylinositol-anchored or transmembrane proteins, and

they act as strong chemorepellents or weak chemoattractants. Semaphorins act through two families of receptors: the neuropilins and plexins. Plexins interact directly with class 1, 2, 4, 5, 6, 7, and V semaphorins, whereas class 3 semaphorins require neuropilin as a coreceptor to signal through plexin.

In the limb, Sema3A and neuropilin-1 signaling are actively involved in motor axon growth and guidance (Huber et al., 2005). In the absence of Sema3A, motor axons enter the limb prematurely, which suggests that Sema3A controls the timing of motor axon ingrowth to the limb. In addition, severe fasciculation defects occur in the spinal nerve in the absence of Sema3, and motor neurons in the LMC exhibit dorsoventral guidance defects at forelimb levels. By contrast, Sema3F and neuropilin-2 signaling guides the LMC (m) neurons into the ventral limb, but they have no function in the regulation of axon fasciculation.

Repulsive responses to semaphorin signaling seems to result from the modification of cytoskeletal components of the growth cone. Intracellular domains of activated plexins initiate a signal transduction cascade that leads to axon repulsion by the simultaneous inhibition of Rac1 and the activation RhoA. Sema3A can induce rapid collapse by the depolymerization of filopodial F-actin, thereby preventing the polymerization of new F-actin bundles and increasing the endocytosis of the Sema3A receptors neuropilin-1 and plexin and the signaling molecule Rac1. Neurite retraction occurs when Plexin-B1–activated Rho-specific GEFs activate RhoA.

G. Ret/Glial Cell-Derived Neurotrophic Factor and EphA4 are Both Required to Drive Axons Dorsally in the Limb

Recent studies show that Ret/glial cell-derived neurotrophic factor is also involved in dorsal axon projections in the limb (Kramer et al., 2006). In Ret mutant mice, the dorsal nerve trunk is significantly reduced, whereas the ventral nerve trunk is enlarged. This is the result of the misprojection of axons into the ventral nerve trunk, which leaves a reduced number of axons dorsally. Ret does not regulate EphA4's expression or vice versa: when Ret or EphA4 is absent, EphA4-positive or Ret-positive axons are now found in the ventral nerve trunk, respectively. It remains to be determined if Ret/glial cell-derived neurotrophic factor acts in concert with or independently of the EphA4/ephrin-A signaling to drive axons dorsally in the limb.

H. Spontaneous Electrical Activity Affects the Guidance and Patterning of Motor Axons in the Hind Limb

In addition to molecules playing a role in axon guidance, rhythmic spontaneous episodes of electrical activity play an essential role in neural development. It has been known for more than a decade that electrical activity plays a role in the refinement of neural connections in the visual system (Katz and Shatz, 1996). However, more recently, it has been shown that electrical activity is also required at earlier stages, when motor axons are making axon pathfinding and innervation decisions. Slowing the frequency of spontaneous rhythmic motor neuron activity by half results in axons remaining in large fascicles when they diverge from the common nerve trunk in the plexus (Hanson and Landmesser, 2004). This is a result of the rapid reduction of the polysialic acid (PSA) moiety, a negatively charged carbohydrate, from the neural CAM (NCAM). Additionally, the slowing and blocking of spontaneous rhythmic

bursting both during the stages of dorsal–ventral pathfinding decisions (stages 24 and 25) and during the later stages of target muscle innervation (stages 28–30) with the chronic treatment of picrotoxin (γ-aminobutyric acid$_A$ receptor antagonist) or strychnine (glycine receptor antagonist) result in the downregulation of EphA4 from LMC(l) motor axons. Thus, it is most likely that it is the alterations of cell adhesion (NCAM) and guidance molecules (EphA4) that cause the errors in dorsal–ventral pathfinding. Recent results where electrical activity is enhanced in motor neurons show that their axons make incorrect choices with regard to the muscles to innervate, but that dorsal–ventral pathfinding is normal (Hanson and Landmesser, 2006).

I. Cell Adhesion Molecules Guide Axons to Their Target Regions

CAMs play an integral role in axon guidance and fasciculation. CAMs are large glycoproteins that are divided into four families: the cadherins, the Ig superfamily, the integrins, and the selectins, with the first three families found in the nervous system. The expression of CAMs varies from broad to discrete, with some cells expressing multiple CAMs on their surface. Although integrins interact with ECM molecules, cadherins and the majority of Ig CAMs undergo homophilic binding with CAMs that are present on adjacent cells, and this leads to cell–cell adhesion.

Often, the Ig superfamily of CAMs is associated with axons, and it plays a dominant role in neurite fasciculation and outgrowth (Doherty et al., 1995). NCAM is a member of this family, with its extracellular domain composed of five Ig-like domain and two fibronectin type III repeats. NCAM homodimers bind to the fibroblast growth factor receptor to promote neurite outgrowth. Additionally, NCAM is capable of binding to L1, TAG-1/axonin-1, and heparan sulfate proteoglycans (Walsh and Doherty, 1997). NCAM is widely expressed during development, although expression becomes restricted to neuronal tissue in adults.

PSA is a linear homopolymer of sialic acid attached to a carbohydrate core that attaches primarily to NCAM (Tang and Landmesser, 1992). PSA is required for the defasciculation of axons, allowing them to respond more readily to guidance cues. During development, this is seen both during the early stages of limb innervation, when the upregulation of PSA on motor axons allows the axons to rearrange into muscle-specific nerve trunks when entering the plexus, and during later stages, when axon bundles separate out to innervate the muscles (Tang and Landmesser, 1993). PSA functions by altering the adhesive interactions of axons with other axons or their muscle targets through steric interference.

L1, which is another member of the Ig superfamily, is a single-pass transmembrane protein with six Ig-like domains and five fibronectin type III repeats in the extracellular domain. Although L1 usually forms homodimers, it is capable of interacting with many other molecules, including integrins, ECM molecules, and ankyrin through its cytoplasmic domain. Studies of animals missing the molecule strongly support a role for L1 in axon guidance. L1 initiates neurite outgrowth in the same way as NCAM by altering levels of intracellular Ca^{2+}. Neurite outgrowth also results from the clustering of either NCAM or L1 via the activation of the mitogen-activated protein kinase pathway (Schmid et al., 1999; 2000).

J. Connecting the Eye to the Brain: Axon Navigation and Topographic Maps

Visual behavior depends on the correct, stereotypical connection of retinal ganglion cells with the next visual processing center, the optic tectum (the superior colliculus in mammals). During development, axons of retinal ganglion cells exit the eye through the optic disc, bundling together to form the optic nerves. In mammals, some axons cross the midline of the hypothalamus at the optic chiasm and extend dorsally in optic tracts to the contralateral side of the brain, whereas some axons remain on the ipsilateral side. Upon reaching the tectum, axons must select from an array of targets and form precise synaptic connections with them (Figure 24.6).

Retinal ganglion cells (RGCs) are the projection neurons of the retina. In visual systems with contralateral and ipsilateral axon projections, RGC axons decide whether to cross the ventral midline at the optic chiasm to project contralaterally or to remain ipsilaterally. EphB and ephrin-B signaling also plays a key role in this event. Initially, the optic chiasm lacks the expression of EphB ligand, which allows EphB-positive axons to cross the chiasm to form

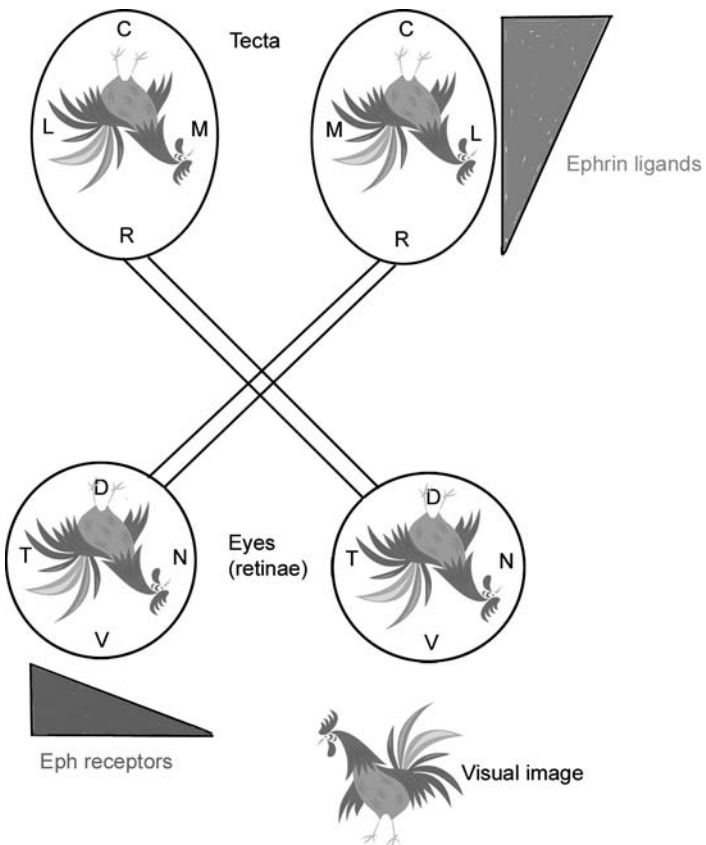

FIGURE 24.6 The organization of visual projections in the retinotectal system and the expression of the Eph family. A chicken in the visual field (visual image) is projected onto each retina so that the image is rotated 180°. Retinal ganglion cells from each retina project the visual image to their first target, the contralateral tectum. In the retina, Eph receptors are expressed high in the temporal *(T)* retina but lower in the nasal *(N)* retina. In the tectum, ephrins are highest in the caudal *(C)* tectum and lowest in the rostral *(R)* tectum. *D,* Dorsal; *V,* ventral; *L,* lateral; *M,* medial. (See color insert.)

contralateral projections. However, ephrin-B2 is upregulated in the chiasm, thereby blocking EphB axons from crossing and forcing them to project ipsilaterally (Williams et al., 2003). The inhibition of ephrin-B2 in mice results in the abolishment of ipsilateral projections. This inhibition at the optic chiasm is likely mediated by EphB1, because the EphB1 null mutation results in a reduction in the number of axons projecting ipsilaterally and an increase in the number of axons projecting to the contralateral portion of the brain.

Eph/ephrin signaling plays an integral role in the guidance and patterning of retinal ganglion cells on the tectum or the superior colliculus (see Figure 24.6). Topographic projections from the nasal–temporal axis of the retina extend to the posterior–anterior axis of the tectum. Previous studies have shown that EphA is highest in the temporal retina and lowest in the nasal region, whereas ephrinA2/A5 expression is highest in the posterior tectum. Axons of nasal retinal ganglion cells that lack EphA innervate the ephrinA-rich posterior tectum, whereas EphA-positive temporal axons innervate the anterior tectum and terminate in a region with low ephrinA expression. Mice that lack ephrins have small defects in the topographic projections of retinal ganglion cells onto the superior colliculus, which suggests that these molecules are required for the accurate patterning of retinal ganglion connections (Nakamoto et al., 1996; Feldheim et al., 2000). However, it is surprising that the topographic maps in the mutant animals are not more defective. These results suggest strongly that other factors are required for these precise topographic maps to form correctly.

K. Activity is Required for the Refinement of Retinal Ganglion Cell Terminal Arbors

The initial topographic innervation of the optic tectum/superior colliculus requires refinement, because it is characterized by low accuracy and some disorganization of the terminal arbors of the axons (Shatz, 1996). Axons innervating inappropriate targets die within a few days, whereas terminal arbors are pared down and remodeled to become more precise (Witte et al., 1996). Spontaneous activity is essential to refining the topographic map. Retinal waves of activity are propagated by the release of acetylcholine by amacrine cells (Stellwagen et al., 1999). Acetylcholine depolarizes RGCs as well as additional amacrine cells. Blocking activity with tetrodotoxin prevents arbor refinement.

III. CELL BIOLOGY UNDERLYING AXON PATHFINDING AND CONNECTIVITY

The growth cone, which is a highly motile structure at the tips of growing axons, acts as the sensor for extracellular guidance cues (Figure 24.7; Kalil and Dent, 2005). Growth-cone motility and guidance responses that cause the axon to advance, turn, or stall are regulated by the actin and microtubule cytoskeleton. Although the understanding of the exact role of the growth cone and its components is just beginning to be fleshed out, future studies will continue to focus on this structure and how it detects and responds to guidance molecules in the local environment.

The growth cone consists of a central domain, which is occupied primarily by bundled microtubules, and the peripheral domain, which contains actin and actin-binding proteins. The peripheral domain is composed of the leading edge of the growth cone, lamellipodia, and filopodia, and it is actively

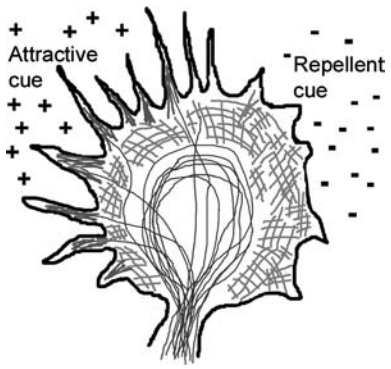

FIGURE 24.7 **The growth cone contains cytoskeletal proteins that are active in response to cues in the environment; a model for the organization and reorganization of the cytoskeleton in the growth cone when it encounters attractive or repellent cues is shown here.** The growth cone contains bundles of microtubules (*thin gray lines* in middle of figure) in its central domain, a meshwork of actin filaments (*dark gray lines* on left side of figure) more laterally, and bundles of actin filaments *(hashed lines)* that penetrate the filopodium. When the growth cone encounters an attractive cue on its left, the number of filopodia is increased, and actin filaments are elongated, thus promoting a turn toward the attractive cue. A repellent cue causes a reduction of actin bundles and a loss of filopodia and dynamic microtubules, which leads to growth-cone repulsion or avoidance.

involved in sensing guidance cues and responding to them. The actin-containing periphery is highly dynamic, often extending and retracting actin-based filopodia and veil-like lamellipodia throughout the axon's navigation to target sites. This dynamism can best be appreciated by tracking growth cone movements over time using live imaging approaches.

Microtubules, which are located primarily in the central domain of the growth cone, have their growing (plus) ends facing toward the periphery. The highly dynamic properties of microtubules enable them to project into both lamellipodia and filopodia. Microtubules play a central role in growth-cone steering, because, when they are inhibited with pharmacologic agents, growth-cone turning is obliterated. The polymerization of actin drives the protrusion of the growth cone's plasma membrane; in this way, it is thought to promote cell motility. How this works on a mechanistic level is unclear. Actin filaments are also involved in growth-cone retraction, and this involves the assembly, movement, and disassembly of actin networks.

Myosin is thought to underlie the retrograde flow of actin in the growth cone and growth-cone retraction; however, the exact roles of myosin are unclear. Recent studies have shown that Sema3A activates myosin in growth cones, thereby providing a link between the extracellular and intracellular domains of the neuron (Gallo, 2006). Inhibiting myosin decreased the retrograde flow of actin significantly, but the overall length of actin bundles associated with filopodia grew (Medeiros et al., 2006). Thus, it becomes evident that myosin is required in growth cones for the regulation of the length of actin bundles and for retrograde flow.

A. Signal Transduction in the Growth Cone

Receptors for many factors (e.g., netrins, Slits, semaphorins, ephrins) signal in growth cones through complicated pathways that converge on the Rho family of small GTPases: Rac, Rho, and Cdc42. Rac and Cdc42 are activated

by attractive cues, and they promote actin polymerization in lamellipodia and filopodia, which leads to growth-cone extension. By contrast, repulsive cues induce Rho to reduce the polymerization of actin and to cause growth-cone retraction. Recent studies have shown that two GEFs (ephexin and Vav2) regulate Rho GTPases and that they are necessary for axon repulsion (Sahin et al., 2005; Cowan et al., 2005). In future studies, it will be necessary to understand how ephrin signaling and other factors influence the actin cytoskeleton by regulating Rho GTPases to promote growth-cone extension or retraction.

B. Protein Synthesis in the Growth Cone

How do growth cones respond so quickly to extracellular cues and environmental signals? Interestingly, the growth cone is thought to undergo local protein synthesis; this is in contract with the prevailing view, which is that protein synthesis happens exclusively in the neuronal cell body. Earlier studies have shown that polyribosomes are present in dendrites adjacent to postsynaptic sites and that mRNAs localize to dendrites (Steward and Fass, 1983; Steward and Levy, 1982). However, the view that axonal protein synthesis occurs has been more controversial. Several mRNAs have been localized to young vertebrate axons (Brittis et al., 2002). Growth cones of developing cortical and hippocampal neurons in culture contain rRNA and poly(A) mRNA. mRNAs have even been identified in growth cones, including mRNAs for actin and the microtubule-associated protein tau (Bassell et al., 1998).

Campbell and Holt (2001) have addressed whether local protein synthesis occurs in growth cones using Xenopus retinal ganglion axons in culture. The application of the guidance cues Sema3A or netrin-1 resulted in a rapid increase in protein synthesis as measured by the incorporation of tritiated leucine (Campbell and Holt, 2001). Using specific antibodies, these authors also showed that ribosomal proteins, capped-RNA, and translation initiation factors were abundant in RGC growth cones. To test whether protein synthesis played an integral role in axon guidance, growth cones were assayed for their ability to turn and collapse in the presence of various inhibitors of protein synthesis (i.e., anisomycin, cycloheximide, and rapamycin). Growth cones typically turn and extend toward a netrin-1 gradient, whereas they turn away from a Sema3A gradient. Importantly, inhibiting RNA translation in isolated axons completely blocked the attractive and inhibitory responses to netrin-1 and Sema3A, respectively.

Slits and their receptors (Robos) are required for many axon pathfinding decisions, including midline guidance. Recent studies have shown that the collapse of axon growth cones in response to Slit requires local protein synthesis and endocytosis (Piper et al., 2006). The exposure of growth cones to Slit results in the activation of regulators of protein translation and mitogen-activated protein kinases. Of note is that Slit treatment caused a fast protein-synthesis–dependent decrease of cytoskeletal actin and a protein-synthesis–dependent increase in cofilin, an actin-polymerizing protein. These findings link guidance cues, the actin cytoskeleton, and local protein synthesis.

IV. CONCLUSIONS

Developmental genetic studies have shown that transcription factors, secreted molecules/cell-surface proteins, and spontaneous activity play

crucial roles in the pathfinding and patterning of axonal connections. How these factors converge on an axonal growth cone and cause it to turn away or move forward is just beginning to be understood. Importantly, the results of these studies have enormous implications for tissue regeneration and harnessing stem cells to remediate behavior or to treat disease. It may be that the molecular and activity cues used during development to guide axons to their final target regions can be reused to regenerate damaged nervous systems. Indeed, great strides have been made in the stem cell field during the past few years; however, the molecules that link neural stem cells to their targets to drive functional behavior, including locomotion and sensory perception, are unknown. Thus, we expect that the results of these developmental genetic studies will exert strong influences on future clinical therapeutics.

SUMMARY

- Axon pathfinding and the patterning of axonal connections involve molecular cues and spontaneous electrical activity. These molecular cues include transcription factors, the Eph family, semaphorins and their receptors, and classical CAMs.
- Multiple receptors and ligands have been identified that function at the level of the plasma membrane. Little is understood about how these factors work mechanistically inside the neuron to direct axon connections.
- Although a wide variety of receptors and ligands are required for axon pathfinding and patterning axonal connections, we know very little about how multiple signals that an axon growth cone receives are integrated during axon pathfinding events.
- Recent studies that focus on the cell biology underlying axon guidance, including cytoskeletal components and protein synthesis, were performed *in vitro*. Few studies have addressed these issues *in vivo*. Future studies will be directed toward understanding the *in vivo* relevance of these processes within the complexity of the native embryonic environment.

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GLOSSARY

Attractive cues
Molecules that are typically synthesized and secreted by other cells in the local environment that influence growth cones in a positive manner by causing them to move toward the source of the attractive cue. These types of cues can be morphogens that are secreted at a distance and that function as chemoattractants, or they can be proteins that localize to the plasma membrane of cells in the environment that require cell–cell contact.

Gradients
Morphogens are often laid down near neighboring cells in a gradient. The gradient of the morphogen is highest at its source, where it is synthesized and secreted, and it is reduced in concentration as one moves away from the source.

Growth cones
The tip of the growing axon of a neuron that is specialized for responding to cues in the environment and that steer the growth cone toward its target region. Growth cones are thought to be the neuron's sensors.

Morphogens
Factors that are typically made at a distance from growth cones and that act as guidance cues. These factors affect the steering of growth cones and likely affect intracellular signaling processes and the growth cone's cytoskeleton.

Repulsive cues
Molecules that are typically synthesized and secreted by other cells in the local environment and that influence growth cones in a negative way, causing them to turn and move away from the source of the repulsive cue. These types of cues can be chemorepellents, or they may be attached to the plasma membranes of cells and require cell–cell contact.

Topographic neural maps
The nervous system often makes topographic maps that reflect the external world. These maps lend precision and organization to the nervous system. For example, neurons in the retina project their axons in a topographic manner onto targets in the brain and reflect the visual world exactly; in addition, motor neurons located at precise positions in the spinal cord project their axons to innervate particular target muscles. One can reliably predict a motor neuron's position in the spinal cord by which type of muscle it will innervate.

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25 RETINAL DEVELOPMENT

KATHRYN B. MOORE and MONICA L. VETTER

Department of Neurobiology and Anatomy, University of Utah, Salt Lake City, UT

INTRODUCTION

The vertebrate neural retina has long been a model for investigating the mechanisms governing the patterning and differentiation of neural tissue. For more than 150 years, the anatomy and physiology of the mature retina have been extensively studied, and this has provided a wealth of information about the organization and function of this tissue. It is a highly specialized extension of the central nervous system that consists of seven principal cell types: (1) rod photoreceptors; (2) cone photoreceptors; (3) bipolar cells; (4) ganglion cells; (5) horizontal cells; (6) amacrine cells; and (7) Müller glial cells. Importantly, many of these major cell classes are further divided into multiple distinct subtypes. For example, up to 22 different subtypes of amacrine cells have been described (MacNeil and Masland, 1998).

The cell types of the mature retina are arranged into three distinct layers that serve specialized functions in the processing and transmitting of information about the visual world (Figure 25.1). The outer nuclear layer is composed of the rod and cone photoreceptors; the inner nuclear layer is composed of horizontal cells, bipolar cells, and amacrine cells; and the ganglion cell layer is composed of retinal ganglion cells and displaced amacrine cells. The cell bodies of Müller glia are within the inner nuclear layer, but the cells traverse all layers and terminate to form two membranes: the external limiting membrane and the inner limiting membrane. The nuclear layers are interconnected through two synaptic layers. Rods and cones synapse onto the dendrites of horizontal cells and bipolar cells in the outer plexiform layer, and bipolar cells and amacrine cells synapse onto the retinal ganglion cell dendrites in the inner plexiform layer. Finally, retinal ganglion cell axons and astrocytes form the nerve fiber layer, which lies closest to the vitreous.

Although the architecture of the retina has been well documented, the challenge for developmental biologists has been to understand how this precise architecture is assembled during development. The retina begins first with specification of the eye field region in the anterior neural plate, and this is followed

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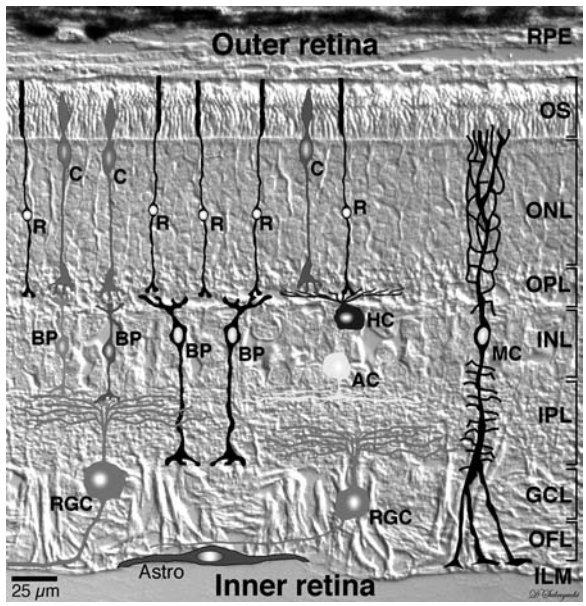

FIGURE 25.1 Organization of the vertebrate retina. Nomarski image of a representative vertebrate retina demonstrating the arrangement of cell types within the laminated retina. There are three nuclear layers: the outer nuclear layer *(ONL)*, the inner nuclear layer *(INL)*, and the ganglion cell layer *(GCL)*. They are separated by two synaptic layers: the outer plexiform layer *(OPL)* and the inner plexiform layer *(IPL)*, which interconnect the nuclear layers. The retinal cell types can be distinguished on the basis of laminar position and morphology within the retina. Rod *(R, black)* and cone *(C, blue, red, green)* photoreceptors are found in the ONL, whereas their outer segments *(OS)* lie adjacent to the retinal pigment epithelium *(RPE)*. The INL contains amacrine cells *(AC, yellow)*, horizontal cells *(HC, dark blue)*, and bipolar cells *(BP, black, green, pale blue)*. Müller glia *(MC)* pass through the entire retinal tissue; their somata reside in the INL, and they terminate in structures called *endfeet* in the GCL and microvilli in the OS. Retinal ganglion cells *(RGC, purple)* and displaced amacrine cells (not shown) reside in the GCL, and axons from the RGCs run through the optic fiber layer *(OFL)*. (Image courtesy of Don Sakaguchi, Iowa State University. See color insert.)

by the evagination of this domain from the walls of the closing neural tube to form the optic vesicle. As the embryo matures, the optic vesicle invaginates to form the optic cup, the inner layers of which will develop into the neural retina. The neuroepithelial cells of the embryonic retina differentiate into the various retinal cell types in a stereotyped overlapping sequence that is conserved across species. In this chapter, we will follow the development of the neural retina from the specification of the eye field and the morphogenesis of the eye through retinal progenitor proliferation and cell fate specification. What will emerge is an appreciation for the role of both extrinsic signals and intrinsic factors in guiding patterning and differentiation throughout retinal development as well as an understanding of how many of the same factors are important for multiple steps of retinal development. Importantly, these same general principles also apply to other aspects of central nervous system (CNS) development.

I. EYE FIELD FORMATION

Specification of retinal fate is a multistep process whereby subsets of embryonic cells undergo gradual restrictions in potential. This process through

which cells acquire differential abilities to contribute to the retina begins early during development, before gastrulation and the formation of the neural plate. Retina-competent precursors are first selected in the cleavage-stage blastomeres on the basis of a lack of genes that specify mesoderm or endoderm (Zaghloul et al., 2005). A group of these cells later becomes biased toward becoming retina-producing precursors, a phase that is dependent on their location within the field of bone morphogenetic protein (BMP) antagonists (Zaghloul et al., 2005). A critical step in retinogenesis is the formation of the true retinal progenitor population; these cells arise from a single bilateral functional domain within the anterior neural plate known as the *eye field* (Figure 25.2; Adelmann, 1929; Wilson and Houart, 2004).

It is now well established that the eye field is the source of retinal progenitors that will give rise to all retinal cell types (Zaghloul et al., 2005). This medially located domain of the anterior neural plate becomes specified over a period of time between late gastrulation and the formation of the open neural plate by signals from the anterior mesoderm. To date, a single specific eye field–inducing factor has not been described, and the emerging picture is that this domain is formed through a series of molecular subdivisions within the anterior neural plate coupled with the sustained expression of a group of transcription factors. Morphogenetic movements are also critical for this process.

A. Extrinsic Factors Regulating Eye Field Formation

Head-inducing factors such as Cerberus and Dkk, anterior-expressing transcription factors such as Otx2 and Lhx1, and Noggin and Notch signaling may play roles in establishment of the eye field (Zaghloul et al., 2005). Other major early contributors likely include Wnt/β-catenin signaling, which is critical for subdividing the anterior neural plate during gastrulation, whereas signals such as BMP can suppress the specification of eye field gene expression (Wilson and Houart, 2004). In some species (e.g., zebrafish), the transcription factor Rx3 may further bias bipotential cells toward an eye field fate (Stigloher et al., 2006). The identity of other molecular signals important for eye field formation as well as an understanding of how these extrinsic signals integrate with intrinsic molecules during gastrulation and early neurulation remains elusive.

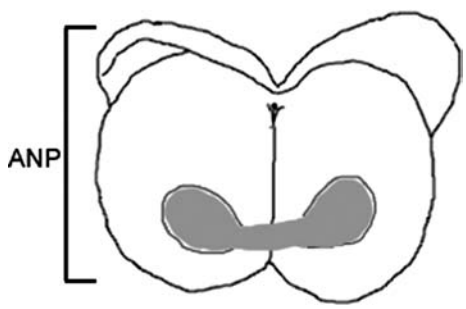

FIGURE 25.2 The eye field is established in the anterior neural plate. Cartoon of a frontal view of the anterior neural plate *(ANP)* of an embryonic day 8 mouse just before neural tube closure and before the eye field has completely separated into two. The approximate location of the eye field is shown bordered in *dark gray*. *Light gray* represents the expression of eye field transcription factors that specify the retinal progenitor population.

B. Eye Field Transcription Factors

Eye field cells in all vertebrates can be molecularly defined by the sustained and overlapping expression of a group of transcription factors called the *eye field transcription factors* (EFTFs), which are required for retinal development. Together these genes comprise the "eye regulatory network," a self-regulating network that specifies the eye field to be retinal progenitors (Zuber et al., 2003). Although their expression appears to be overlapping, Zuber and colleagues (2003) demonstrated that these factors in fact are expressed in nonidentical domains, which suggests that the historical eye field is in fact composed of smaller subdivisions characterized by different combinations of gene expression. As mentioned previously, the upstream signaling pathway and growth factors that initiate the expression of these EFTFs after neural induction and that set up these subfields are presently unknown, although Wnt signaling is required for the maintenance of several of these EFTFs (Maurus et al., 2005). Each of these genes—*ET, Lhx2, Pax6, Optx2, Six3, Rx1-Rx3,* and *Tll*—has been shown to be critical for eye development (Chow and Lang, 2001). For example, the overexpression of *Pax6* causes the formation of ectopic eye structures in several species, whereas *Rx1*-mutant mice lack eyes (Gehring and Ikeo, 1999). Mutations in the human orthologues of these genes often result in microphthalmia or anophthalmia (Graw, 2003).

C. Morphogenetic Movements

An important step in the process of eye field formation is the correct positioning of progenitors within the gastrula and the early neurula. These progenitors undergo morphogenetic movements that enable them to receive fate-directing signals from the local environment. Progenitors that fail to integrate properly into the eye field may not be able to initiate or maintain the expression of EFTFs. There are two major morphogenetic movements that affect eye field formation, and extrinsic as well as intrinsic signals are important for these events. First, during gastrulation, cells must move into the correct position within the anterior neural plate such that they may express EFTFs. To accomplish this movement, progenitor cells must disperse into the eye field while remaining coherent. Two signaling pathways have been identified that are important in these processes. Anterior neural plate cells and eye field progenitors demonstrate cellular dispersal during gastrulation (Wilson and Houart, 2004). In the frog, this behavior is modulated by ephrinB1, which signals through its extracellular domain by interacting with Dishevelled and making use of the Wnt–planar cell polarity pathway (Lee et al., 2006; Moore et al., 2004), whereas fibroblast growth factor (FGF) signaling negatively regulates this progenitor dispersal (Moore et al., 2004). Zebrafish Wnt11 acting through Fz5 regulates the cohesion of eye field progenitor cells, whereas Wnt11's activation of noncanonical Wnt signaling may limit the extent of the eye field by preventing posteriorizing signals from affecting retinal progenitors (Cavodeassi et al., 2005). Although it is known that ephrinB1's effects on eye field formation do not require Wnt11 (Lee et al., 2006), the precise mechanisms coordinating progenitor dispersal and coherence are not known. Downstream of Wnt and ephrin signaling, EFTFs are also able to promote cellular movements into the eye field (Kenyon et al., 2001; Moore et al., 2004).

The second major morphogenetic event in eye field formation is the division of the initially continuous single eye field into two bilateral eye primordia. This separation, which occurs at the end of gastrulation, is mediated by midline-derived Sonic hedgehog (Shh) signaling, which represses the expression of EFTFs (Wilson and Houart, 2004). Shh mutations cause severe cyclopia in mice and humans, whereas increased Shh results in the loss of eyes in some species and suppression of retina in others. However, Shh signaling alone may be insufficient for eye field separation. In fish, midline neuroectodermal cells migrate anteriorly, physically separating the retinal fields, and this process requires nodal signaling (Wilson and Houart, 2004). It is not clear whether the nodal-dependent movement of ventral diencephalic precursors is also required in other species for eye field separation.

II. OPTIC VESICLE FORMATION

After the eye field has been established, morphogenesis of the eye begins with the evagination of optic vesicles from the walls of the developing forebrain (Chow and Lang, 2001). This process begins early as the edges of the neural plate lift up to initiate neural tube closure. Some EFTFs (e.g., *Rx*) are required for these initial stages of eye morphogenesis. For example, there is a no optic vesicle formed in zebrafish that are mutant for *Rx3,* because retinal progenitor cells fail to initiate optic vesicle evagination (Stigloher et al., 2006). As the optic vesicles evaginate, they come into contact with the overlying surface ectoderm, which has already been induced to form the lens during the early embryonic stages. This prelens ectoderm thickens to form the lens placode, and the optic vesicles then invaginate to form the bilayered optic cups (Chow and Lang, 2001). The outer layer of the optic cup will become retinal pigment epithelium (RPE), whereas the inner layer will thicken and become the neural retina. The optic stalk forms ventrally and connects the optic cup with the ventral forebrain. In response to ventral signals, the optic vesicle and the optic stalk invaginate ventrally to form the optic fissure, which is important for the exit of retinal axons and for the formation of the hyaloid artery, which supplies blood to the retina (Chow and Lang, 2001). Factors such as *Pax6* and *Lhx2* are required for the initial formation of the optic cup; in *small eye (sey)* embryos, which are mutant for *Pax6,* the optic vesicle forms but fails to progress and form an optic cup (Gehring and Ikeo, 1999).

A. Extrinsic Signals Pattern the Optic Vesicle

The next step, which is the invagination of the optic cup, depends on initial contact between the optic neuroepithelium and the prelens ectoderm, and there is evidence that this depends on BMP signaling (Hyer et al., 2003). Such interactions between the neuroepithelium of the optic vesicles and adjacent tissues (e.g., the overlying surface ectoderm and the surrounding mesenchyme) are important throughout the process of eye morphogenesis, and they are particularly critical for the regional specialization of the optic vesicle into the neural retina, the RPE, and optic stalk domains (Figure 25.3; Yang, 2004). For example, FGF secreted by the lens ectoderm is required to induce neural retina, and it coordinates the placement of the neural retina domain at the distal tip of the optic vesicle (Hyer et al., 1998). In chick optic

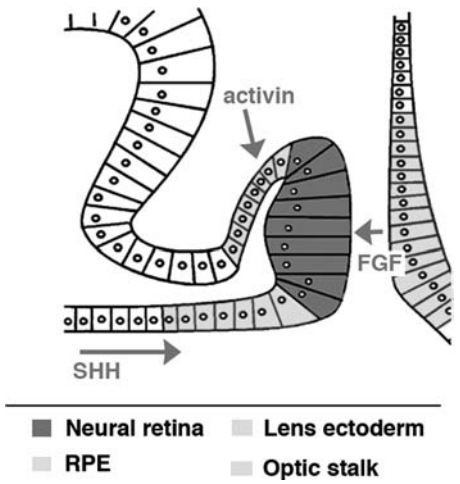

FIGURE 25.3 **The optic vesicle is patterned by extrinsic signals from surrounding tissues.** Cartoon of the optic vesicle stage of eye development. As the optic vesicle forms, it receives signals from adjacent tissues that pattern the optic vesicle into distinct subdomains. For example, fibroblast growth factor from prospective lens ectoderm will induce neural retina at the distal part of the optic vesicle, whereas signals from extraocular mesenchyme, such as activin-like signals, are required to form the retinal pigment epithelium *(RPE)*. Ventral midline signals, such as sonic hedgehog *(Shh)*, pattern the optic vesicle along the proximal-distal axis (relative to the midline), thereby establishing more proximal structures, such as the optic stalk and ventral RPE.

vesicle explants, ectopic FGF is sufficient to convert prospective RPE to a neural retina fate, whereas blocking FGF2 signaling using neutralizing antibodies prevents neural retina differentiation (Pittack et al., 1997). RPE formation depends on signals from extraocular mesenchyme, because coculture of this tissue with optic vesicle explants promotes RPE development. Alternatively, RPE fails to develop in the absence of extraocular mesenchyme. Activin can substitute for extraocular mesenchyme, which suggests that transforming growth factor beta (TGFβ)–like signals may be mediating these effects during normal development (Fuhrmann et al., 2000). Hedgehog acts from the ventral midline to pattern the optic vesicle along the proximal–distal axis (relative to the midline), thereby establishing more proximal structures (e.g., optic stalk, ventral RPE) as distinct from the neural retina (Yang, 2004).

B. Intrinsic Factors Define Tissue Compartments Within the Optic Vesicle

In response to these patterning events, the different subdomains of the optic vesicle are characterized by the expression of characteristic genes that maintain the distinct tissue identities. For example, the RPE layer of the optic cup expresses the basic helix–loop–helix (bHLH) zipper gene *Mitf,* which is required to maintain RPE identity. In *Mitf* mutant mouse eyes, dorsal portions of the RPE transdifferentiate into neural retina (Martinez-Morales et al., 2004). Similarly, the homeodomain transcription factor gene *Chx10* is expressed in the neural retina domain in a manner that is mutually exclusive with *Mitf*. Loss of *Chx10* in mice results in the transdifferentiation of retinal progenitor cells into RPE and in the upregulation of *Mitf* and other

pigment-specific genes, which suggests that *Chx10* is required for the maintenance of the neural retina fate (Horsford et al., 2005; Rowan et al., 2004). The optic stalk expresses yet another set of genes that are important for the development of this tissue, including *Pax2* and *Vax1*. In the mouse, *Pax2* initially overlaps with *Pax6* expression in the optic vesicle at early stages of eye development, but this ultimately resolves through mutual repression into a sharp boundary between *Pax2* expression in the optic stalk and *Pax6* expression in the optic cup (Schwarz et al., 2000).

C. Axial Patterning of the Neural Retina

In addition to the basic subdivision of the optic vesicle into neural retina, RPE, and optic stalk, there is patterning along the dorsal–ventral (DV) extent of the retina itself, which has significance for the correct topographic projection of retinal axons onto the tectum. Specifically, dorsal retinal ganglion cell axons project to the ventral tectum, and ventral axons project to the dorsal tectum; this is under the control of complementary gradients of EphB receptors in the retina and ephrinB ligands in the tectum (McLaughlin and O'Leary, 2005; see Chapter 24). DV patterning of the retina is established by Shh signaling from the ventral midline and by BMP4 signaling from the dorsal optic vesicle/cup, which is also opposed by ventroptin (a BMP antagonist that is expressed in the ventral optic vesicle; McLaughlin and O'Leary, 2005). This mirrors the role of ventral Shh and dorsal BMP signaling in establishing a DV pattern throughout the developing central nervous system (Lupo et al., 2006). Gradients of Shh and BMP signaling within the optic vesicle result in DV differences in gene expression, such as that of *Vax2,* which is expressed in the ventral retina, and that of *Tbx5,* which is expressed dorsally (McLaughlin and O'Leary, 2005). The dorsal misexpression of Shh suppresses BMP4 expression and expands ventral markers into the dorsal retina, whereas a loss of Shh causes the expansion of BMP4 expression into the ventral retina and a loss of ventral markers. Thus, Shh and BMP4 act in an opposing manner to establish DV patterning within the developing retina (McLaughlin and O'Leary, 2005). The misexpression of *Tbx5* and *Vax2* genes causes defects in eye morphogenesis and abnormal visual projections, which suggests that these genes are important for maintaining DV spatial information within the developing optic cup (Leconte et al., 2004). Other factors, such as retinoic acid and FGF, may also contribute to DV patterning, and they may cooperate with Shh and BMP4 to establish DV gradients of gene expression within the retina (Yang, 2004).

In addition to DV patterning, there is also nasal–temporal (NT) patterning of the retina that results in opposing gradients of ephB expression in the retina and ephrinA expression in the tectum; however, less is known about how this is established (McLaughlin and O'Leary, 2005). In zebrafish, FGF signals from the telencephalic primordium promote nasal and suppress temporal retinal cell fates (Picker and Brand, 2005). During the early stages of some species, the winged-helix transcription factor BF-1 marks the nasal half of the optic vesicle and the optic stalk, whereas BF-2 is restricted to the temporal half (Hatini et al., 1994); however, whether they are required for NT differences within the retina is not clear. *Pax6* also appears to play a role in the development of polarity along both the DV and NT axes of the retina, and it regulates both *Tbx5* and *Vax1/2* expression (Leconte et al., 2004).

III. CONTROL OF RETINAL GROWTH

After the optic cup has been subdivided into different tissue domains and patterned, there is a period of sustained growth. The control of proliferation and cell number is central to the development of any tissue, because it can determine the ultimate size and cellular composition of that tissue. In the developing neural retina, cell cycle regulation ensures that appropriate numbers and proportions of differentiated cells are generated and that progenitors are maintained in sufficient number to last through the entire period of retinal histogenesis. In addition, cell cycle control is intimately tied with cell fate, because the birth date of a cell (i.e., when it permanently exits the cell cycle) is a key factor in the determination of the cell type that is generated.

Retinal progenitor proliferation can be controlled in a number of different ways, including through intrinsic factors such as cell cycle regulators and transcription factors, by secreted factors and their transmembrane receptors, and by modulating factors that control progenitor maintenance and symmetric versus asymmetric cell divisions. It is likely the interplay between these different modes of regulation that determines whether a cell proliferates, how rapidly it does so, and, ultimately, when it exits the cell cycle. Although we may generalize, there is also increasing evidence for the heterogeneity of progenitors, with different factors playing a role in distinct subsets of cells.

A. Cell Cycle Regulation in Retinal Progenitors

The cell cycle is divided into four phases: (1) the S-phase, during which DNA is replicated; (2) the G2 phase, which is a checkpoint for DNA replication errors in preparation for mitosis; (3) the M phase, when cells undergo mitotic cell division; and (4) the G1 phase, which is a period of cell growth and a checkpoint phase during which signals can influence cell cycle exit or progression. Specific cell cycle regulators act during each of these phases to regulate the proliferation of retinal progenitors, and components that act during the G1 phase are particularly important for determining whether cells exit or continue to cycle and how rapidly they do so (Dyer and Cepko, 2001; Levine and Green, 2004). For example, cyclin D1 is expressed in retinal progenitors, and it is required to promote normal G1 progression by activating cyclin-dependent kinases (e.g., *Cdk4* or *Cdk6*), which phosphorylate retinoblastoma family proteins, thereby allowing cells to progress. Mice that are null for cyclin D1 have reduced retinal progenitor cell proliferation and hypocellular retinas; clearly, however, other factors also play a role, because there is still some proliferation (Levine and Green, 2004). In addition to these positive regulators, cell cycle progression is constrained by Cdk-inhibitors that can modulate the cell cycle rate or promote exit. For example, the Cdk-inhibitor Kip1 can force cells out of the cell cycle, and the protein accumulates in cells as they become postmitotic. Kip1 null mice show prolonged ectopic retinal progenitor proliferation, and this finding is consistent with a role for Kip1 in the promotion of the cell cycle exit during retinal histogenesis (Dyer and Cepko, 2001; Levine and Green, 2004). The related protein Kip2 plays a similar role in a subset of embryonic retinal progenitor cells. Other key cell cycle regulators include the E2F family of proteins, which are transcription factors that are essential for the G1 to S phase transition, and retinoblastoma proteins, which constrain E2F activity and thus regulate G1 progression (Levine and Green, 2004).

Together, these factors act in a coordinate manner to regulate cell cycle dynamics in retinal progenitors, and they are often influenced by mitogenic signals that modulate their activity.

B. Transcription Factors Regulating Retinal Progenitor Proliferation

Multiple homeodomain transcription factors are expressed in the developing optic vesicle and the progenitors of the neural retinal, including *Rx, Pax6, Six3, Six6, Chx10,* and *Prox1* (Levine and Green, 2004). These factors are important for determining the regional identity of developing retinal tissue, and they also contribute to the regulation of progenitor proliferation, although the mechanisms are not always well defined. One example is *Chx10,* which is expressed in retinal progenitors throughout eye development. Natural "ocular retardation" mouse mutants were identified based on their microphthalmic phenotype, and the retardation was shown to be the result of mutations in *Chx10* (Burmeister et al., 1996). These mutant mice had reduced retinal cell numbers and a severe defect in retinal progenitor cell proliferation as a result of a prolonged G1 phase. This defect can be compensated for by a combined inactivation of Kip1, which restores progenitors to a more normal rate of proliferation (Green et al., 2003). Thus, *Chx10* may directly modulate components of the cell cycle machinery to regulate progenitor proliferation.

Another transcription factor with a demonstrated role in retinal progenitor proliferation is *Six6* (*Optx2* in Xenopus). When this factor is overexpressed in Xenopus, embryos develop with giant eyes as a result of the enhanced proliferation of retinal progenitor cells (Zuber et al., 1999). In mice, *Six6* is expressed in retinal progenitor cells during the early stages of retinal development, and a loss of *Six6* results in premature cell cycle exit and retinal hypoplasia. *Six6* may act to maintain the proliferation of retinal progenitors through the transcriptional repression of *Kip1,* because *Six6* forms a complex with Dach corepressor proteins and binds to the *Kip1* promoter (Li et al., 2002). Consistent with this is the fact that *Kip1* is upregulated in the *Six6* mutant retina, and this presumably leads to progenitor cell cycle exit.

Sox2 is a POU-homeodomain transcription factor that is expressed in progenitors throughout the developing CNS. Although it is not a classic eye field homeodomain transcription factor like *Pax6* or *Chx10,* it is particularly important in the developing retina. Loss-of-function studies in both Xenopus and mouse reveal that *Sox2* is required for maintaining neural competence in the developing retina and for normal retinal progenitor proliferation (Taranova et al., 2006; Van Raay et al., 2005). When *Sox2* is inhibited, progenitors continue to proliferate, but they progress more slowly. Although *Sox2* is expressed throughout the CNS, the requirement for this gene in retinal development is most pronounced as a result of the absence of related family members *Sox1* and *Sox3,* which elsewhere act redundantly with *Sox2*. Future studies will need to address how these transcription factors interface with the cell cycle machinery to control retinal progenitor proliferation.

C. Regulation of Progenitor Proliferation by Extrinsic Factors

Retinal progenitors respond to a number of extrinsic cues (e.g., secreted or membrane-bound factors) that can directly influence their mitotic activity. For example, in the mouse retina, Shh is expressed by retinal ganglion cells (RGCs) soon after the mice are born, which signals to adjacent precursor

cells in the neuroblast layer to promote their proliferation (Figure 25.4; Dakubo and Wallace, 2004). Mice with a conditional deletion of Shh in RGCs (Thy1-Cre; $Shh^{-/c}$) have smaller retinas, exhibit reduced progenitor proliferation and reduced cyclin D1 expression, and show premature retinal neuron differentiation, thus depleting the retinal progenitor pool (Wang et al., 2002). Retinal progenitor cells can be isolated and grown in culture, and, under these *in vitro* conditions, Shh can stimulate retinal progenitor proliferation and suppress RGC differentiation. Thus, Shh derived from RGCs is an important signal for maintaining retinal progenitors and for promoting their proliferation in the developing mouse retina (Dakubo and Wallace, 2004).

In other parts of the developing nervous system, Wnt signaling acts through the canonical β-catenin pathway to promote progenitor proliferation by regulating the expression of cell cycle regulators such as cyclin D1 and c-myc. In the developing chick retina, Wnt2a has been proposed to maintain retinal progenitors by suppressing retinal neuron differentiation and promoting their proliferation (Van Raay and Vetter, 2004), although there is conflicting evidence that suggests that Wnt signaling instead promotes peripheral ciliary body fates in the developing eye (Cho and Cepko, 2006). Thus, additional studies will be needed to resolve how Wnt signaling regulates retinal cell proliferation.

In vitro and *in vivo* studies have provided evidence that other secreted factors also influence retinal precursor cell proliferation, including epidermal growth factor (EGF), dihydroxyphenylalanine, BMP, and insulin-like growth factor 1 (Dyer and Cepko, 2001). Early studies showed that FGF was a potent mitogen for retinal progenitor cells at younger embryonic stages, whereas older progenitors were more responsive to TGFα (Lillien and Cepko, 1992). Thus, numerous extrinsic signals can act as mitogens and contribute to the regulation of proliferation and cell cycle exit during retinal development, and these signals may change over developmental time.

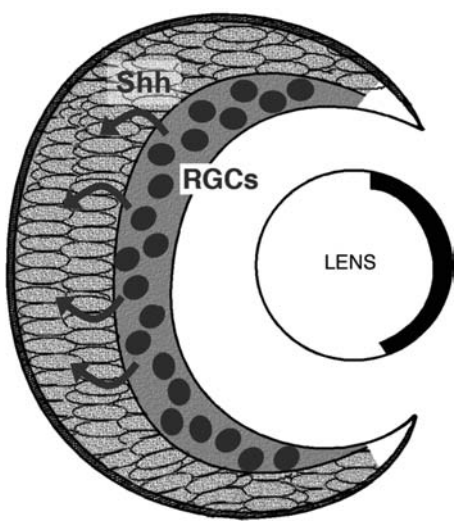

FIGURE 25.4 **Shh regulates retinal progenitor proliferation.** Cartoon of a section through the embryonic mouse eye. Sonic hedgehog *(Shh)* is expressed by retinal ganglion cells *(RGC)* soon after they are born, which signals to adjacent retinal progenitor cells in the neuroblast layer to promote their proliferation.

D. Factors Influencing Progenitor Maintenance

Additional factors clearly contribute to overall retinal cell number. As will be discussed later, the decision of a cell to differentiate depends on the balance between the factors that promote cell cycle exit and differentiation and the factors that oppose this and constrain differentiation, such as components of the Notch signaling pathway (Louvi and Artavanis-Tsakonas, 2006). These inhibitory pathways are essential for maintaining a progenitor population throughout development. For example, blocking Notch signaling allows progenitors to differentiate and exit the cell cycle prematurely, and this results in the depletion of the progenitor pool and a reduced retinal cell number at the end of development (Perron and Harris, 2000a).

Progenitors also undergo different modes of cell division that control the orientation of cell division. Progenitors can divide symmetrically to generate two identical daughter cells, thereby expanding the progenitor pool. Alternatively, progenitors can divide asymmetrically, with one daughter cell becoming postmitotic and differentiating while the other daughter remains a progenitor and continues to divide. The balance between symmetric and asymmetric divisions determines the balance of proliferative expansion versus differentiation within the retina, and it plays an important role in the timing of cell genesis and thus cell-type specification (Cayouette et al., 2006). In many parts of the CNS, symmetric divisions occur in the plane of the neuroepithelium, whereas asymmetric divisions have an apical–basal orientation. However, there is evidence from the developing zebrafish retina that all divisions are within the plane of the neuroepithelium parallel to the ventricular surface, with symmetric divisions being in the central–peripheral orientation and asymmetric divisions being in the circumferential orientation (Das et al., 2003). The molecular mechanisms controlling asymmetric cell division appear to be evolutionarily conserved, because Inscuteable, which regulates the asymmetric division of Drosophila neuroblasts, controls spindle orientation and cell fate in the developing mammalian retina (Zigman et al., 2005).

E. Regulation of Cell Death

Throughout the developing CNS, the cell number is regulated not just through proliferation but also through apoptotic cell death, which eliminates neurons that are produced in excess during development. There is evidence for cell death in least two stages of retinal development. The first period of retinal cell death occurs during early ocular morphogenesis. For example, in mice, apoptotic cells are evident within the optic neuroepithelium at the optic vesicle and optic cup stages (Laemle et al., 1999). In the chick, retinal cell death within the optic cup coincides with the onset of neuronal birth and migration, and it is dependent on the nerve growth factor secreted by microglia, which migrate into the retina during development (Frade and Barde, 1998). Nerve growth factor is known to bind to the p75 neurotrophin receptor and to trigger intracellular apoptotic pathways. Alternatively, brain-derived neurotrophic factor maintains the survival of newborn RGCs (Frade et al., 1997). Later (and coincident with target innervation), there is a second period of retinal neuron loss through cell death (Vecino et al., 2004). Although multiple retinal cell types have been shown to undergo cell death, this has been most carefully documented for retinal ganglion cells, because 50% to 90% of RGCs that are born eventually die. During this period, RGCs are less

dependent on brain-derived neurotrophic factor, so whether there is a specific survival factor responsible for maintaining RGCs during the target innervation period remains unclear. Possible candidate survival factors include NT-3 and NT-4, which can promote the survival of differentiated RGCs in chick and rat, respectively (Vecino et al., 2004).

IV. RETINAL CELL FATE SPECIFICATION

Retinal progenitors within the optic cup will give rise to six major neuronal cell types (rod receptors, cone photoreceptors, bipolar cells, amacrine cells, horizontal cells, ganglion cells) and to Müller glia. The time at which a cell undergoes its final mitotic division and permanently exits the cell cycle is referred to as the "birth date" of the cell, and it is highly coupled with retinal cell fate. Retinal cell birth dating studies have been performed in multiple species in which 3H-thymidine is incorporated into DNA to label cells that are undergoing their final S phase. These studies have revealed that retinal cell types are born in a sequence that is largely conserved across species (Figure 25.5). Retinal ganglion cells are always the first retinal cell type to appear as the optic cup forms, with cone photoreceptors and horizontal cells also being born early and bipolar cells, rods, and Müller glia being generated last (Livesey and Cepko, 2001). However, the birth dates of the various cell types are highly overlapping, and the proportions of each cell type can vary widely across species. Because multiple cell types can be generated at any

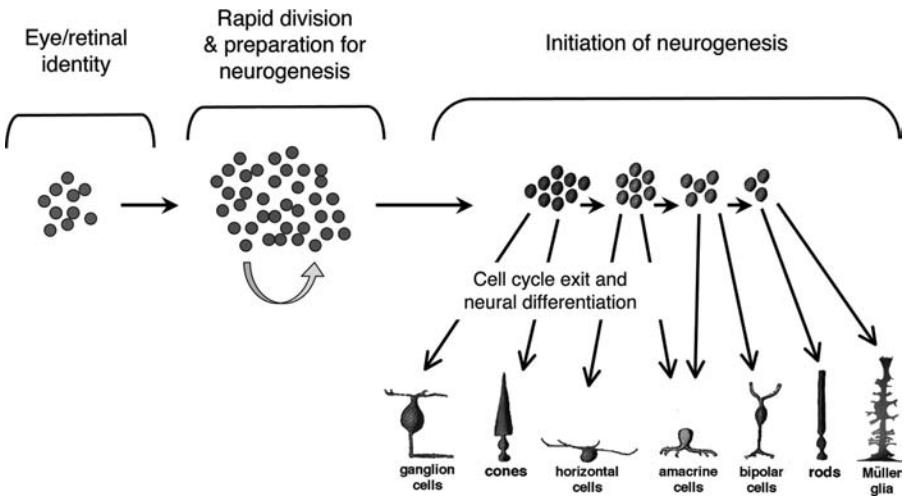

FIGURE 25.5 **Retinal cell types are generated from multipotent progenitor cells.** Retinal progenitors first acquire regional identity in the eye field and the optic vesicle as defined by the expression of a combination of homeodomain transcription factors. Progenitors then undergo proliferative expansion and prepare for the onset of neurogenesis. The periods for proliferation and cell fate determination are overlapping and tightly coupled. The cell types of the retina are generated in an overlapping sequence that is conserved across species. Retinal progenitors are multipotent, ultimately giving rise to all of the neuronal cell types of the retina as well as to Müller glia. However, at any one time, they appear to give rise to a limited subset of retinal cell types, which suggests that the intrinsic competence of retinal progenitors changes over developmental time *(indicated by changes in color)* so that at a given time they can produce one or a few retinal cell types (Cepko et al., 1996). (See color insert).

given developmental time, this suggests that birth date is not the sole determinant of retinal cell fate.

Within the retina, there are spatial gradients of differentiation. For example, in rodents, differentiation in the central retina precedes that in the peripheral retina. The spread of differentiation has been beautifully documented in the zebrafish retina, where neurogenesis initiates in a ventral patch and then spreads in a fanlike manner circumferentially around the retina, with later cell types being generated in successive fanlike waves that follows the initial spread of ganglion cell genesis (Hu and Easter, 1999). As these various retinal cell types are generated, they migrate to assume their final position within the developing retina, and they begin to establish the laminated architecture of the retina.

A. Retinal Progenitors Are Multipotent

An early question was whether there are dedicated progenitors for each retinal cell type or whether progenitors are multipotent. This was addressed by performing lineage analysis by injecting individual progenitor cells with a tracer or by infecting them with retroviruses and tracking the fate of labeled cells (Altshuler et al., 1991). This analysis showed that progenitors give rise to radial clones of cells that populate all three layers of the retina and that they can include multiple classes of neurons as well as Müller glia. However, the composition of the clones was highly variable, with no fixed combinations of retinal cell types being generated; this indicates that retinal progenitor cells are multipotent rather than being dedicated to the generation of specific retinal cell types. Retroviral infections during later developmental stages showed that there is an increasing bias toward later-born cell types, which suggests that there may be progressive restriction in the developmental potential of retinal progenitors.

Although progenitors are clearly multipotent, at any one time they appear to give rise to a limited subset of retinal cell types. For example, heterochronic transplants or *in vitro* culture experiments have shown that progenitors from one developmental stage placed into a new environment will adopt the fates that are appropriate for the original developmental stage (Livesey and Cepko, 2001). This has led to the competence model of retinal development first proposed by Cepko and colleagues (1996), which suggests that the intrinsic competence of retinal progenitors changes over developmental time so that at a given time progenitors can produce one or a few retinal cell types (see Figure 25.5).

B. Retinal Progenitor Heterogeneity

Although retinal progenitors as a population are multipotent, there is evidence that not all progenitors are equivalent. The heterogeneity of progenitors is evident from variability in marker gene expression and from the expression of different cell cycle regulators in different subsets of retinal progenitors. For example, the cdk inhibitor $p57^{Kip2}$ is upregulated in just 15% of retinal progenitors that exit the cell cycle from embryonic days 14.5 through 17.5 of mouse development, and this subset is distinct from the cells that upregulate the related cdk inhibitor $p27^{Kip1}$ (Dyer and Cepko, 2001). Molecular heterogeneity may reflect dynamic changes in the competence of retinal progenitors, or it may indicate intrinsic differences between progenitors with respect to what retinal cell type will be generated.

How do retinal progenitors change over time, and how does this affect their competence to generate different retinal cell types? It is known that progenitors change in their cell cycle kinetics over time, with cell cycle length increasing throughout development (Alexiades and Cepko, 1996). The mode of cell division changes, with early divisions biased toward divisions that give rise to two mitotic progenitors; later divisions are more likely to generate postmitotic daughters (Dyer and Cepko, 2001). There are also changes in EGF receptor expression, which determines the response of progenitors to mitogenic EGF signals. However, it remains an unresolved question precisely how the intrinsic competence of retinal progenitors changes and what the molecular basis for these changes might be. Parallels have been drawn between retinal development and neuroblast development in Drosophila, where there is the sequential expression of transcription factors that regulate the competence of these neuroblasts to sequentially give rise to distinct neuronal types (Pearson and Doe, 2004). It will be interesting to see whether related mechanisms are at work in the developing vertebrate retina and to what extent extrinsic signals modulate these changes in competence states.

C. Basic Helix–Loop–Helix Factors Regulate Retinal Neurogenesis

As retinal progenitors prepare to differentiate, what are the molecular mechanisms governing retinal cell fate decisions? As is does for much of development, retinal histogenesis relies on the interplay of extrinsic signaling pathways and intrinsic factors that act to coordinate cell cycle exit and retinal cell differentiation. bHLH transcription factors are critical intrinsic regulators of retinal neurogenesis (Vetter and Brown, 2001). During nervous system development, these factors play an important role in promoting neural fates and in inhibiting glial fates, and they are thus called *proneural factors*. In the retina, the misexpression of proneural bHLH factors promotes the neuronal differentiation of retinal progenitors and suppresses the Müller glial cell fate (Vetter and Brown, 2001). These factors also coordinate cell cycle exit and differentiation, and they are required for retinal progenitors to exit the cell cycle at the appropriate developmental time.

bHLH transcription factors are expressed in retinal progenitor cells in a pattern that suggests that they function to regulate the successive stages of retinal neurogenesis. In both zebrafish and chick. *Ath5* expression spreads in a wave across the developing retina, and this immediately precedes a corresponding wave of RGC differentiation (Vetter and Brown, 2001). In the mouse. *Math5, NeuroD, Ngn2, Math3,* and *Mash1* are expressed in spatial and temporal patterns that correspond with the birth dates of different retinal neuron classes (Table 25.1). *Math5* is expressed first, just before the onset of RGC differentiation, and this is followed by *Ngn2* and *Ath3,* then *NeuroD,* and, finally, *Mash1*. In Xenopus, proneural bHLH factor expression is highly overlapping, but posttranslational mechanisms regulate bHLH factor activity, thereby ensuring that they act at different stages of retinal histogenesis (Moore et al., 2002).

In a way that is consistent with their patterns of expression and activity, proneural bHLH factors are required for the development of specific classes of retinal neurons. The disruption of *Ath5* in both the mouse and the zebrafish has revealed that it is essential for RGC development, whereas other bHLH factors regulate the differentiation of additional retinal cell types

TABLE 25.1 Retinal Proneural Gene Expression in Mouse (Hatakeyama and Kageyama, 2004; Vetter and Brown, 2001)

Proneural Genes	Expression Window	Role in Retinal Cell Type
Math5	E11- P0	Retinal ganglion cells
Ath3	E12.5; and E16-postnatal	Horizontal, amacrine, and bipolar cells
Ngn2	E12.5 through E16.5, gone by P0	?
NeuroD	E13.5-E18- persists in postnatal retina	Amacrine cells, cone and rod photoreceptors
Mash-1	E14.5- PN14	Bipolar cells, cone and rod photoreceptors

(Hatakeyama and Kageyama, 2004; Vetter and Brown, 2001). For example, the loss of *NeuroD* expression in mice causes a delay in amacrine cell development, but if both *NeuroD* and *Math3* are disrupted, then there is a complete loss of amacrine cell differentiation, which suggests that these two bHLH factors act redundantly to regulate amacrine cell genesis (Hatakeyama and Kageyama, 2004). Similarly, *Mash1* and *Math3* both contribute to bipolar cell development, and the disruption of both results in the loss of virtually all bipolar cells (Hatakeyama and Kageyama, 2004).

Although it is clear that specific bHLH factors are required for the development of different classes of retinal neurons, overexpression studies suggest that they may not strictly determine retinal neuron type. For example, in the mouse, although *Mash1* and *Math3* are implicated in bipolar cell differentiation, their overexpression in the early postnatal retina promotes the differentiation of rod photoreceptors rather than bipolar cells. Furthermore, *NeuroD* can generate either amacrine cells or photoreceptors, depending on when it is misexpressed (Hatakeyama and Kageyama, 2004). In Xenopus, the overexpression of *Ath5* in early progenitors can promote an RGC fate at the expense of later-born cell types; however, if it is misexpressed later, it promotes later-born cell types (Moore et al., 2002). These studies reveal that the role of bHLH factors in cell-type specification depends in part on developmental context and that additional factors are required.

D. Other Factors Contribute to Retinal Cell Fate Specification

Homeodomain transcription factors have also been implicated as key regulators of retinal cell fate. Multiple homeodomain factors are expressed in the developing retina, including *Pax6, Rx, Otx2, Prox1, Six3, Six6 (Optx2),* and *Chx10 (Vsx1),* and most are expressed first in retinal progenitors and then in a subset of differentiating and postmitotic retinal cells. For example. *Chx10* is expressed in retinal progenitors throughout eye development and then later in postmitotic bipolar cells; in *Chx10* mutant mice, bipolar cells fail to differentiate (Burmeister et al., 1996). Although *Chx10* is required for bipolar cell genesis, the misexpression of *Chx10* alone is insufficient to direct cells to adopt the bipolar cell fate. However, the coexpression of *Chx10* with the bHLH factors *Mash1* or *Math3* promotes a specific increase in bipolar cell differentiation (Hatakeyama and Kageyama, 2004). These findings have led to the proposal that homeodomain transcription factors cooperate with proneural bHLH factors to create a combinatorial code for each retinal cell type

(Hatakeyama and Kageyama, 2004). For example, *Pax6* and *Six3* can cooperate with *NeuroD* or *Math3* to promote amacrine and horizontal cell genesis, although neither alone is sufficient to do so. It remains to be determined to what extent this combinatorial code model applies to the determination of other retinal cell types.

There are also examples of genes that are expressed in retinal progenitors that differ from *Chx10* and that are both necessary and sufficient to direct progenitor cells to adopt specific retinal cell fates. For example, *Prox1* is present in differentiating horizontal, bipolar, and AII amacrine cells. *Prox1* mutant mice show defects in progenitor proliferation, but they also lack horizontal cells, whereas the misexpression of *Prox1* in postnatal progenitor cells promotes horizontal cell formation (Dyer et al., 2003). Similarly, the *Foxn4* winged helix/forkhead transcription factor is required for most amacrine neurons and all horizontal cells to be specified, whereas the overexpression of *Foxn4* strongly promotes an amacrine cell fate. *Foxn4* regulates the expression of *Math3, NeuroD1,* and *Prox1,* which are important factors in the differentiation of amacrine and horizontal cells (Li et al., 2004). Thus, although a combinatorial code of bHLH factors and homeodomain factors may contribute to the genesis of some cell types, additional complexity and levels of regulation are likely involved in normal retinal cell fate determination.

E. Negative Regulators of Retinal Neurogenesis

Negative regulatory mechanisms are also involved in governing the development of the retina. The transmembrane protein Notch can function through cell–cell interaction to negatively regulate neuronal differentiation and cell fate determination in response to the ligands, such as Delta or Serrate, that may be expressed on neighboring cells (Louvi and Artavanis-Tsakonas, 2006). Upon ligand binding, the Notch receptor is proteolytically cleaved, and it releases the intracellular domain, which translocates to the nucleus and interacts with the CSL (CBF1, Su(H), LAG-1 family) transcription factor to activate the expression of effector genes, including bHLH repressors of the Hairy/Enhancer of split family. There is a clear role for the Notch signaling pathway in the negative regulation of retinal cell differentiation (Perron and Harris, 2000a). For example, the overexpression of an activated form of Notch in Xenopus retinal precursor cells causes the inhibition of retinal cell differentiation, whereas blocking Notch signaling causes cells to differentiate early and to adopt early retinal cell fates (Perron and Harris, 2000a). There is evidence in mice that Notch can act at multiple stages of retinal development: disrupting Notch signaling in early retinal progenitors leads to increased cone differentiation, whereas disruption at later stages results in enhanced rod photoreceptor production (Jadhav et al., 2006).

The activation of the Notch signaling pathway initiates the expression of the repressors *Hes1* and *Hes5,* which can antagonize the expression or activity of proneural bHLH genes and thus inhibit retinal neurogenesis (Louvi and Artavanis-Tsakonas, 2006). For example. *Hes1* is important for preventing the premature onset of retinal neurogenesis in mouse, and it appears to do so by inhibiting *Math5* expression (Lee et al., 2005). The sustained expression of *Hes1* or *Hes5* not only suppresses retinal neuron differentiation but also promotes Müller glial cell differentiation, which suggests that progenitors that lack proneural activity adopt the glial fate (Vetter and Moore, 2001).

F. Extrinsic Signals Can Modulate Retinal Neurogenesis

In addition to Notch/Delta signaling, additional factors have been shown to modulate retinal cell fate decisions. In zebrafish, FGF signaling from the optic stalk is both necessary to activate *Ath5* expression in the retina and to initiate retinal neurogenesis near the optic stalk. As RGCs begin to differentiate, they produce Shh, which promotes the proliferation of progenitors and acts as a negative feedback signal to suppress further RGC differentiation (Dakubo and Wallace, 2004). The secreted factor GDF11 is also expressed by differentiating RGCs, and it acts to control the numbers of differentiating RGCs, amacrine, and photoreceptor cells by controlling the duration of expression of *Math5* (Kim et al., 2005). Other factors regulate the genesis of other cell types, such as the TGFβ family member activin A, which affects photoreceptor differentiation both *in vitro* and *in vivo* (Yang, 2004). It will be important to determine how extrinsic signals modulate intrinsic factors to influence retinal cell fate decisions.

G. Regulation of Postmitotic Retinal Cell Differentiation

After retinal neurons have been specified, there are specific genes that are expressed in early differentiating cells that are required for the execution of programs of differentiation within these cells. For example, during photoreceptor development, the transcription factors *Crx, Nrl,* and *Nr2e3* all regulate aspects of terminal differentiation. The *Crx* paired-type homeobox transcription factor regulates the expression of multiple photoreceptor-specific genes, and it is required for the normal development of photoreceptor outer segments (Furukawa et al., 1997). *Nrl* and *Nr2e3* work together with *Crx,* and they play critical roles in controlling rod versus cone development. *Nrl* is required for rod development, and *Nrl* mutant mice show a loss of rods and in increase in S cones (Mears et al., 2001). *Nr2e3* acts downstream of *Nrl* to suppress the expression of cone-specific genes, and *Nr2e3* mutant mice have hybrid photoreceptors that express both rod and cone genes (Chen et al., 2005; Corbo and Cepko, 2005; Peng et al., 2005). This suggests that cells may first choose to become photoreceptors and then undergo either cone or rod differentiation.

During RGC development, the class IV POU homeodomain transcription factor *Brn3b* is expressed downstream of *Ath5* in early postmitotic and mature RGCs, and it regulates the expression of the genes required for the terminal differentiation of RGCs (Mu and Klein, 2004). In *Brn3b* mutant mice, normal numbers of RGCs are born, but these neurons show defects in axon outgrowth, and they undergo apoptotic death before *birth,* which suggests that *Brn3b* is not required for initial RGC specification but that it does regulate critical aspects of differentiation, maturation, and survival. Gene expression analysis of *Brn3b* mutant retina reveals that only a subset of genes normally expressed in RGCs depend on *Brn3b* for their expression (Mu and Klein, 2004). Other transcription factors, such as the *Dlx1/Dlx2* and *BarH* homeodomain factors, are also expressed in differentiating RGCs, and they play crucial roles in that differentiation (de Melo et al., 2005; Poggi et al., 2004). It is likely that, for most cell types, the full terminal differentiation program depends on transcriptional regulation by multiple transcription factors that act downstream of the initial cell fate decision.

V. MÜLLER CELL GENESIS

Müller cells are specialized radial glial cells that are present in the retinae of all vertebrate species. As the major macroglial cells of the retina, Müller glia span the entire retina and form a link between neurons and retinal spaces. These highly specialized cells serve as differentiated support cells, and they have numerous roles throughout retinal development, in the mature retina, and in pathologic situations (Bringmann et al., 2006). Mature Müller cells maintain homeostasis in the retinal microenvironment and thus provide support for neurons, protect against oxidative stress, and regulate neurotransmitter uptake and neuronal signaling (Bringmann et al., 2006). In pathologic situations, Müller glia may at first play a protective role for retinal neurons, but they can then undergo reactive gliosis and have longer-term detrimental effects (Bringmann et al., 2006). During development, Müller glia are important for retinal organization and for connecting retinal neurons to other retinal compartments. In vascularized retinae, young Müller cells are involved in retinal vascularization and in the formation of the blood–brain barrier (Bringmann et al., 2006).

A. Mechanisms Governing the Differentiation of Müller Glia

Retinal neurons and Müller glia are formed from common multipotent precursors, and the glia are the last major cell type to be generated during retinal histogenesis (Livesey and Cepko, 2001). There are three broad mechanisms that might explain why Müller cells are the last retinal cells to differentiate. First, the differentiation of Müller glia may be delayed such that, by late development, progenitors adopt the sole remaining fate by default. A second mechanism suggests that the genetic program controlling neurogenesis may be actively suppressed, thus allowing progenitors to adopt glial fates. Finally, there may be an instructive genetic program whereby glial-specific genes drive progenitors down a glial path. It is also possible that all three (or more) of these mechanisms are important for the neuron choice as compared with the glial choice. It is clear that the glial fate (like the neuron fate) is determined by a combination of extracellular signals and intracellular mediators that act both positively and negatively on progenitors. In contrast with neurogenesis, although progress has been made in identifying some of the specific molecular components involved in retinal gliogenesis, how these signals and mediators integrate temporally and spatially to generate Müller glia from retinal progenitors is not well understood.

B. Notch Signaling Promotes Müller Glial Fate

Notch, which is a contact-mediated signaling system, has been implicated in the control of glial differentiation (Vetter and Moore, 2001). Notch expression is upregulated in differentiating Müller glia in *Xenopus laevis,* mouse, and rat (Perron and Harris, 2000a), and the activation of Notch signaling can promote glial development (Vetter and Moore, 2001). Notch is important for the timing of retinal differentiation, and it is able to delay or prevent neuronal differentiation (Perron and Harris, 2000a). Notch signaling has been shown to inhibit both the expression and/or the function of bHLH proteins, which are transcription factors that are important for neuronal fate

determination (Perron and Harris, 2000a). Because there is good evidence in the nervous system and in the retina that bHLH factors suppress gliogenesis, one important role for Notch and its effectors in gliogenesis may be to inhibit bHLH expression and/or function. Finally, Notch may act instructively to promote gliogenesis. One possible mechanism involves the activation of negatively acting bHLH factors such as the Notch effectors *HES* and HES-related genes that are expressed in Müller glia and induced by Notch (Vetter and Moore, 2001). The misexpression of both *HES1* and *HES5* in rodent retinae or explant cultures *(HESr2)* promotes Müller gliogenesis, whereas embryos deficient for *HES5* or *HES1* show reduced numbers of Müller glia and increases in retinal neurons (Lee et al., 2005; Vetter and Moore, 2001). These genes may upregulate the transcription factors that promote gliogenesis (as demonstrated in CNS progenitors), and they may also repress neurogenic bHLH factors. Enhanced Notch signaling in the developing zebrafish retina blocks neuronal differentiation and causes cells to cell autonomously adopt glial fates, which suggests an instructive role for Notch in gliogenesis (Scheer et al., 2001).

All of these observations suggest that the Notch pathway may affect gliogenesis by integrating timing, the inhibition of the neurogenic program, and the activation of a glial program. Consistent with this idea is the fact that the inhibition of Notch signaling in late retinal stem cells biases their progeny to increase neuronal markers and to decrease glial markers, whereas activating Notch signaling increases the number of cells expressing glial markers (James et al., 2004). This gliogenic effect of Notch signaling on late progenitors may be related to the ability of Notch to activate the promoters of glial-specific markers in a context-dependent manner. One proposed mechanism for this differential effect is chromatin remodeling. The expression of Brahma, which is an ATPase chromatin remodeling enzyme, is correlated with late retinal histogenesis, and it is known to interact with CSL and the Notch intracellular domain (James et al., 2004). One model is that Brahma (or other components of SWI/SNF chromatin remodeling complexes) interact with Notch, directing it to promoters of glial-specific genes. Understanding the upstream regulation of Notch signaling in the retina and the precise molecular mechanisms of Notch action is important for future investigation.

C. Extrinsic Signals Regulating Glial Development

Several extracellular signals have been identified that either promote or inhibit Müller glia genesis both *in vitro* and, more recently, *in vivo*. EGF promotes the proliferation of Müller glia *in vitro* (Lillien, 1995), whereas, *in vivo*, EGF may be important for the timing of Müller differentiation (Close et al., 2006). Several ligands for EGF receptors are expressed during retinal development, including TGFα and heparin-binding EGF, which is upregulated in patients with proliferative vitreoretinopathy (Close et al., 2006). Other growth factors that may affect glial development include FGFs, TGFβ2, and ciliary neurotrophic factor (Close et al., 2006). Although the strongest data support a proliferative role for these growth factors, the current growth factor data argue that extrinsic signaling pathways may be important for controlling the timing of Müller glia proliferation and differentiation in the developing retina and for helping maintain glial mitotic quiescence in the uninjured mature retina.

D. Intrinsic Factors Required for Müller Glial Differentiation

One important class of intrinsic factors is the cyclin-dependent kinase inhibitors (CKIs), which respond to growth inhibitory signals in the extracellular environment by promoting cell cycle arrest (Vetter and Moore, 2001). Members of a subfamily of CKIs have been shown to regulate Müller cell proliferation and to play a role in Müller glial development. One member of this family, $p27^{kip1}$, is expressed in Müller glia in the mature rodent retina, which suggests that it plays a role in the mature phenotype of these cells, whereas Müller glia in $p27^{kip1}$ knockout mice are disorganized, exhibit abnormal proliferation, and express glial fibrillary acidic protein (Vetter and Moore, 2001). In some animals, CKIs may play a role in normal gliogenesis. The overexpression of $p27^{Xic1}$ in retinal progenitors in both frog and rodent caused an increase in Müller glia at the expense of later-born neurons, which provides evidence that this gene has an instructive role in retinal gliogenesis (Vetter and Moore, 2001). These factors may also be an important link integrating extracellular signals with other intrinsic mediators, because Notch can synergize with $p27^{Xic1}$ in the regulation of Müller glial differentiation. CKIs may also interact with extracellular signals that regulate proliferation, such as EGF and TGFβ.

VI. ADULT RETINAL STEM CELLS

During embryonic development, the cells of the neural retina are derived from multipotent progenitors that proliferate rapidly, that give rise to the complement of neural cell types, and that are ultimately depleted. Thus, the neurogenic capacity diminishes in most vertebrate organisms as they reach adulthood, with the exception of teleost fishes and urodele amphibians. In these species, there are self-renewing adult stem cells at the margins of the retina in a region known as the *ciliary marginal zone* (CMZ). This proliferative zone contributes to the normal growth of the retina and RPE after the initial embryonic period, and it can also repopulate cells in response to damage or injury (Perron and Harris, 2000b). Within the CMZ, the most peripheral cells have the characteristics of stem cells, because they are slowly dividing, and they only become labeled with prolonged [^{3}H] thymidine labeling. Cells that are more centrally located divide more rapidly and can be labeled with short pulses of [^{3}H] thymidine, which suggests that these are rapidly cycling progenitors (Perron and Harris, 2000b). Lineage tracing experiments in *Xenopus laevis* retina showed that cells in the peripheral region of the CMZ are multipotent and that they can give rise to all major retinal cell types as well as to nonneural pigment epithelial cells (Wetts et al., 1989). The *in situ* analysis of gene expression at the margins of fish and Xenopus retinas also showed that there is a peripheral-to-central sequence of gene expression that recapitulates the temporal sequence of gene expression during retinal histogenesis (Perron et al., 1998).

In adult fish, the cells of the CMZ give rise to all retinal cell types except rods, which are instead derived from a dedicated rod precursor population that is distributed along the entire central-to-peripheral plane of the mature retina and found in the outer nuclear layer interspersed with mature photoreceptors (Otteson and Hitchcock, 2003). These cells are rapidly dividing; they do not self-renew, and they differentiate exclusively into rods, although they

have the potential to become multipotent after injury to the retina. Upon physical ablation or severe neurotoxic damage in fish, retinal neurons and glia are replaced, and new cells arise in part from the rod precursor population (Otteson and Hitchcock, 2003). There appears to be another population of more slowly dividing cells in the inner nuclear layer that is self-renewing, that gives rise to the rod precursors throughout the life of the animal, and that may represent a rod precursor stem cell population.

In the chick, a proliferative population of cells also exists at the margins of the retina for up to 3 weeks after hatching. These dividing cells differentiate and give rise to amacrine, bipolar cells, and Müller cells but not to ganglion cells, horizontal cells, or photoreceptors. However, the injection of insulin and FGF2 into the vitreous chamber of posthatch chickens can stimulate the production of retinal ganglion cells, which suggests that the microenvironment can determine whether these cells differentiate into all retinal cell types (Perron and Harris, 2000b).

A. Mammalian Retinal Stem Cells

In mammals, although there is no classic CMZ, there are cells from the pigmented epithelium of the ciliary body that can, under certain conditions, differentiate into retinal neurons in culture. Dissociated cells from the pigmented ciliary epithelium of mouse, rat, bovine, or human can be grown in culture and stimulated to proliferate by treatment with the mitogen FGF2. At very low frequency, they form nestin-positive neurospheres, which are colonies of neural stem-like cells that have the capacity for self-renewal. A subset of single pigmented cells from dissociated neurospheres gives rise to new neurosphere colonies when recultured, and this can be repeated for at least six generations. These neurospheres are multipotent, because they give rise to cells with retinal progenitor-like properties that can differentiate into retinal neurons or glia expressing markers for rods, bipolar cells, and Müller glia, although markers for retinal ganglion cells, horizontal cells, and amacrine cells are not seen or are extremely rare (Tropepe et al., 2000). However, to date, there is no evidence that cells from the mammalian ciliary epithelium give rise to neurons or contribute to the regeneration of the retina *in vivo*.

B. Müller Cells as a Source of Retinal Stem Cells

One major area of recent investigation concerns whether Müller cells can serve as a source of regenerating neurons for treating retinal degenerative disorders. Although it would appear that Müller glia are not neural stem cells (at least under normal conditions), Müller cells can dedifferentiate, reenter the cell cycle, and proliferate in response to damage (Fischer, 2005). Müller glia have been investigated as a source of neural regeneration, because they can be forced to reenter the cell cycle, downregulate glial specific enzymes, dedifferentiate, express some progenitor and bHLH markers (Fischer, 2005), and/or acquire characteristics of retinal stem cells (Raymond et al., 2006). However, there is no evidence that these cells can differentiate into mature photoreceptors in culture whether stimulated by damage, by combinations of growth factors, or by the overexpression of bHLH transcription factors (Fischer, 2005). Whether future studies can define the conditions necessary to generate fully differentiated neurons that can integrate with and contribute to a functioning retinal circuitry from Müller glia remains to be seen.

VII. CONCLUSIONS AND FUTURE DIRECTIONS

The study of retinal development is built on a foundation of classic anatomic and embryologic studies. However, in recent years, there has been an explosion of knowledge in this area fueled by advances in molecular biology and developmental genetics. There is now an appreciation for the importance of genetic pathways in the regulation of key events during eye development, some of which are conserved from flies to humans (Gehring and Ikeo, 1999). In some cases, this has led to fundamental insights into the causes of human eye disorders (Graw, 2003). Clearly, we do not yet know the whole spectrum of factors that are present *in vivo* that influence cell fate, so much remains to be learned.

As biomedical research continues its rapid pace and new methodologies are developed, there is the promise of solving the fundamental puzzles of how patterning, proliferation, and differentiation are governed throughout retinal development and of how the precise architecture of the mature retina is ultimately generated. In the long term, insights gained from such basic research will significantly advance our efforts to use retinal stem cells or progenitor cells for treating retinal degenerative disease.

SUMMARY

- The eye field is established in the anterior neural plate, and it is defined by the expression of multiple eye field transcription factors.
- The optic vesicle is patterned by extrinsic signals that establish the neural retina domain and generate DV and NT patterns.
- Retinal progenitor cells proliferate extensively under the control of cell cycle regulators, transcription factors, and mitogenic signals.
- Postmitotic retinal cell types are generated from retinal progenitor cells in an overlapping sequence that is conserved across species.
- Retinal progenitor cells are multipotent, giving rise to all retinal cell types; however, they change in their competence over developmental time.
- Multiple transcription factors appear to act in concert to regulate retinal cell fate decisions.
- Inhibitory signals act to maintain a pool of progenitors, to control the number and timing of neuron generation, and to promote the nonneural Müller glial fate.
- In some species, retinal stem cells are maintained as a population of cells at the margins of the retina.

GLOSSARY

Cell birth date
The time at which a cell undergoes its final mitotic division and permanently exits the cell cycle.

Extrinsic factors
Extracellular molecules such as secreted signaling proteins and their transmembrane receptors that provide a cell with information about its environment.

Eye field
A domain in the anterior neural plate that is specified to give rise to eye tissue. This domain expresses a combination of eye field transcription factors that are essential for eye formation.

Intrinsic factors
Intracellular molecules such as cell cycle regulators, transcription factors, and target genes that affect the properties of the cell in which they act.

Neural retina
The sensory neuroepithelium of the eye that contains multiple neuronal cell types organized into three cellular layers. This tissue is part of the central nervous system, and it is specialized for transducing light information and transmitting it to the brain.

Optic vesicle
An evagination of the eye domain from the lateral walls of the embryonic forebrain that will give rise to eye tissues, including the neural retina and retinal pigment epithelium.

Retinal progenitor cell
A dividing cell that will give rise to multiple cell types in the neural retina but that is limited in potential and not capable of prolonged self-renewal.

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RECOMMENDED RESOURCES

Webvision: http://webvision.umh.es/webvision/—A Web site with detailed information about the anatomy and function of the retina and visual system.
Mouse retina SAGE library: http://itstgp01.med.harvard.edu/retina/—Serial analysis of gene expression (SAGE) is a method for the comprehensive analysis of gene expression patterns. This searchable Web site contains SAGE data along with a large number of *in situ* hybridization images from two published studies. One identifies photoreceptor-enriched or specific genes (Blackshaw et al., *Cell* 2001), and the other describes genes that are temporally regulated in development (Blackshaw et al., *PLoS Biol* 2004).
Sernagor E, Eglen S, Harris B, Wong R: *Retinal development*, Cambridge, UK, 2006, Cambridge University Press—A very recent comprehensive book focusing on the molecular, cellular, and physiologic mechanisms governing vertebrate retinal development.

26 NEURAL CREST DETERMINATION

ROBERTO MAYOR

Department of Anatomy & Developmental Biology, University College London, London, United Kingdom

INTRODUCTION

Although the neural crest is a discrete and transient structure that comprises only a few cells, it embodies many of the crucial issues of developmental biology. Questions related to embryonic induction, morphogenesis, differentiation, and even evolution have to be addressed as part of the understanding of neural crest development. The neural crest is formed at the border of the neural plate (Figure 26.1). How is this group of cells induced at that particular position? During and after the closure of the neural tube, the neural crest cells start one of the most dramatic morphogenetic events during early embryogenesis: they are segregated from the neuroepithelia, and they migrate throughout the embryo. How is this segregation from the epithelia and cellular migration controlled? Is it similar to what happens during tumor progression and metastasis? The neural crest differentiates into a prodigious array of cell types (Figure 26.2). How is this differentiation controlled? What determines the fate of each of the neural crest derivatives? Finally, the neural crest represents a key tissue in vertebrate evolution, because many of the typical features of vertebrates (e.g., the jaw) are neural crest derivatives. How did this small group of cells emerge during evolution, and how was it able to play such an important role in vertebrate evolution? These are questions that have fascinated developmental and evolutionary biologists for more than a century.

The neural crest has sometimes been referred to as the fourth germ layer (the first three layers being the ectoderm, the endoderm, and the mesoderm). The crest gives rise to cells that are typical of ectoderm (e.g., neurons, epidermal cells) as well as those with mesodermal characteristics (e.g., muscle, cartilage). The cell types include the following (see Figure 26.2): (1) the neurons and glial cells of the sensory, sympathetic, and parasympathetic nervous system; (2) the epinephrine-producing (medulla) cells of the adrenal gland; (3) the pigment-containing cells of the epidermis; (4) many of the skeletal and connective tissue components of the head; and (5) some of the connective tissue of the heart.

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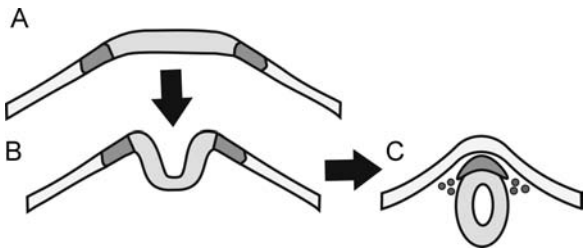

FIGURE 26.1 **Neurulation and neural crest formation. A,** Open neural plate. **B,** Neural folds rise at the border of the neural plate. **C,** The neural tube closes and the neural crest remains between the dorsal neural tube and the epidermis, from where they start their migration. *Light gray,* Neural plate/tube; *dark gray,* neural crest; *white,* epidermis.

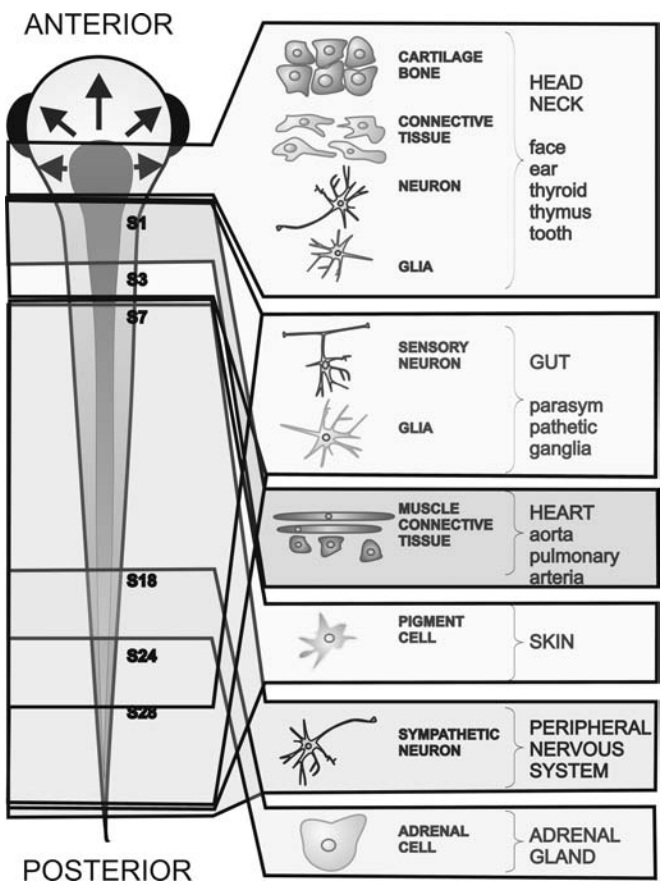

FIGURE 26.2 **Neural crest derivatives**. The central nervous system with the neural crest (*purple*) attached in its dorsal region is represented on the left; numbers indicates somites (anterior to the top, posterior to the bottom). The five panels to the right represent the different kinds of cells derived from the neural crest and the tissues and organs that they form. (See color insert.)

The neural crest was discovered in the chick in 1868 by Wilhelm His. His identified a band of cells lying between the neural tube and the epidermal ectoderm as the origin of the spinal and cranial ganglia; he called this band *Zwischentrang* or *intermediate cord*. The term *neural crest* was used for the first

time by Arthur Milnes Marshall in 1879, according to Brian Hall (1999). Since then, considerable progress has been made in the neural crest field. The cellular and molecular mechanisms that control neural crest induction and differentiation are now beginning to be unraveled. A number of genes that are expressed specifically in the neural crest have been identified and their function studied. Many of the molecules that control neural crest migration have been discovered. We will describe some of these findings in this chapter, but first we will describe some experimental approaches to the study of the neural crest.

I. TECHNIQUES TO IDENTIFY NEURAL CREST DEVELOPMENT

One of the requisites to the study of neural crest cells is the ability to identify them; however, this was a serious problem for many years. Neural crest cells do not exist as a typical tissue, because, very soon after they are induced, they disperse into individual, highly migratory cells. After they reach their final target, they differentiate into a huge variety of different kinds of cells. Three general methods have been used to identify and study neural crest cells:

(1) *The destruction or ablation of neural crest cells.* With this technique, the neural fold or the prospective neural crest is removed or destroyed, and the deficiencies in the embryos are analyzed. This kind of technique was widely used in amphibian and chick studies, and it contributed to the identification of neural crest derivatives. However, it has a major drawback: the extirpation of the neural fold can trigger a regulatory mechanism in the embryo and the regeneration of neural crest precursors from the surrounding tissues.

(2) *Labeling techniques.* (2.1) *The quail-chick markers system.* This technique was developed by Nicole LeDouarin in 1969, and it is based on the ability to distinguish the nucleus of quail cells from chick cells. Quail neural fold cells are grafted in the neural fold of a chick embryo, and the migration and fate are analyzed later by identifying the quail cells in the chick host. A complete and accurate fate map of the neural crest was developed with the use of this technique, and most of the neural crest derivatives were traced back to the neural folds of chick embryos. (2.2) *Dye labeling.* Vital dye staining has long been used to study neural crest migration and differentiation. Several lipophilic dyes have been recently developed, such as DiI and DiO. With this technique, the dyes are applied *in vivo* to the premigratory neural crest cells, and migration and cell fate can then be studied. Other cell lineage markers of common use are lysinated fluorescein, rhodamine dextran, and, more recently, green fluorescent protein.

(3) *Endogenous neural crest markers.* Some enzymes and antigens are expressed more or less specifically in neural crest cells, such as acetylcholinesterase and the antigen HNK-1. However, a true revolution came with the identification of the gene products present specifically in neural crest cells, which can be analyzed by *in situ* hybridization. The first of those genes was identified almost 10 years ago, and it codes for members of the *Snail* gene family (Nieto et al., 1994; Mayor et al., 1995). Since then, several other genes (mostly transcription factors) have been identified as specific markers for the neural crest. Many of these factors are expressed during all of the steps of neural crest development, induction, migration, and differentiation, and they can be used as unequivocal markers for these different processes.

II. SPECIFICATION OF NEURAL CREST CELLS

A. Embryologic Studies of Neural Crest Specification

The neural crest is specified at the border of the neural plate, between the neural plate and the epidermis. Thus, many different tissues are adjacent to the neural crest, and they can be potential sources of inductive signals. The tissues that are in close apposition to the neural crest are the underlying mesoderm, the epidermis, and the neural plate (Figure 26.3). Two kinds of experiments can be used to identify the tissue that induces the neural crest: (1) the tissue can be removed mechanically or genetically, and the development of the neural crest can be analyzed; or (2) the tissue can be added to a competent ectoderm, and the induction of neural crest can be assayed.

Pioneer experiments in amphibians showed that the mesoderm underlying the neural crest has the ability to induce neural crest cells (Raven and Kloos, 1945). Raven and Kloos (1945) dissected different pieces of mesoderm from a neurula amphibian embryo and grafted them into the cavity of a gastrula. After the gastrula developed, the neural crest derivatives were identified by their morphology. They found that medial pieces of mesoderm (dorsal mesoderm or notochord) induce neural plate and neural crest, but that a specific induction of neural crest was obtained when more lateral (paraxial and intermediate) mesoderm was grafted. More recent experiments

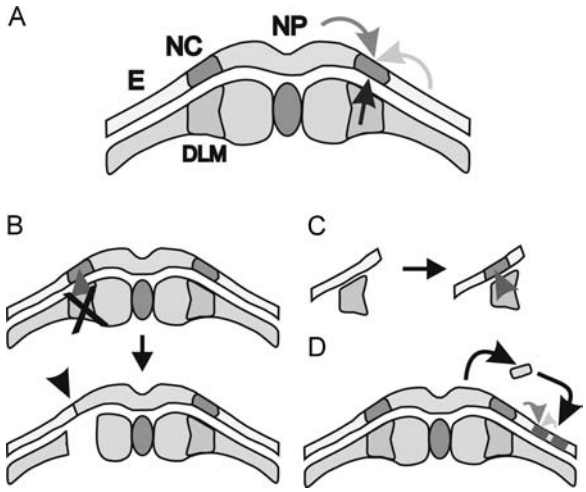

FIGURE 26.3 **Tissues involved in neural crest induction. A,** Left side: tissues surrounding the prospective neural crest (NC): neural plate (NP), epidermis (E), and dorsolateral mesoderm (DLM). Right side: signals that are able to induce neural crest are generated from DLM, from the neural plate (NP), and from the epidermis (*light gray arrow*). **B,** Ablation of DLM. DLM has been ablated by microdissection in amphibian embryos; as a consequence of this ablation, no neural crest develops on the operated side of the embryo. **C,** The induction of neural crest by DLM. Conjugates of competent ectoderm and DLM are able to induce neural crest in the ectoderm. **D,** The induction of neural crest by neural plate/epidermis interaction. Neural plate is dissected from a neurula embryo and grafted into lateral epidermis. Neural crest is induced at the border of the graft by signals coming from the surrounding epidermis and from the grafted neural plate.

have confirmed this early observation. In amphibians, when dorsolateral marginal zone (prospective dorsolateral mesoderm) is removed from the embryo, no neural crest develops (Figure 26.3, A; Bonstein et al., 1998; Marchant et al., 1998). If the same dorsolateral marginal zone is conjugated with competent ectoderm, a strong induction of neural crest markers and some neural crest derivatives (e.g., melanocytes) are observed (Figure 26.3, B; Bonstein et al., 1998; Marchant et al., 1998; Monsoro-Burq et al., 2003). Interestingly, the dorsolateral marginal zone is fated to underlie the neural crest, and this is the same tissue that Raven and Kloos (1945) described as *neural crest inducer.* However, it has been recently shown in zebrafish that the involution of the mesoderm is not required for neural crest induction (Ragland and Raible, 2004). Several zebrafish mutants, such as the *Oep,* do not develop dorsal mesoderm, and the more lateral mesoderm does not involute; thus, because these mutants do not have the mesoderm underlying the neural crest, they are the equivalent of a genetic ablation of this mesoderm. It seems that neural crest is still induced in those mutants; therefore, the mesoderm does not have to underlie the neural crest to induce it. It should be pointed out that, although the dorsal mesoderm is greatly reduced in these mutants and there is no involution of the remaining mesoderm, they still have lateral mesoderm that is capable of sending its inductive signals. In conclusion, a specific dorsolateral mesoderm plays an important role in neural crest induction. The inductive activity of this mesoderm starts before this tissue is involuted, and it continues at least until it is underlying the neural crest.

The other tissues that are adjacent to the neural crest are the neural plate and the epidermis, and it has been shown than an interaction between these two tissues is able to induce neural crest cells. Grafts of neural plate cells into epidermis induce neural crest cells at the border of the graft in amphibian and chick embryos (Figure 26.3, C; Moury and Jacobson, 1989; Selleck and Bronner-Fraser, 1995; Mancilla and Mayor, 1996). By using lineage markers in the grafted neural plate, it was possible to determine that the neural crests induced at the border originated from the neural plate and from the epidermis. Therefore, it seems that signals from the epidermis are able to transform neural plate cells into crest cells and that signals from the neural plate do the same in the epidermal tissue. *In vitro* cocultures of neural plate attached to epidermis also exhibit the expression of neural crest markers, which indicates that this interaction of the neural plate and the epidermis is sufficient to specify the neural crest cells (Selleck and Bronner-Fraser, 1995; Mancilla and Mayor, 1996).

In conclusion, the neural crest is induced by a combination of signals coming from three different sources (see Figure 26.3, *right side*). An interaction between the neural plate and the epidermis changes the fate of these two tissues, causing them to become neural crest cells. In addition, signals from the lateral mesoderm are also involved in neural crest induction. The order in which these signals work to induce neural crest is not known, and it is not known whether they work in parallel or in sequence. The observation that it is possible to induce neural crest by the simple juxtaposition of neural plate and epidermis, without mesoderm, suggests that the inductive activity of the mesoderm could have a role in reinforcing this initial induction setup by the interaction between the neural plate and the epidermis.

B. Molecular Studies on Neural Crest Specification

1. Inductive Signals

The search for the neural crest inductive signals has been long but very fruitful. Five different families of extracellular inductive molecules have been found to be involved in neural crest induction: the bone morphogenetic proteins (BMPs) and its antagonists, like Noggin and Chordin; different Wnts; several fibroblast growth factors (FGFs); retinoic acid (RA); and Notch/Delta.

For all of these molecules, two general kinds of experimental approaches have been used:

(1) *Loss-of-function experiments.* The putative inducer is inhibited, and the induction of the neural crest markers is assayed. The inducer can be inhibited by specific chemical inhibitors that are added to the medium in which the embryos are grown or in which the prospective isolated neural crest are cultured. Another common method of blocking the inductive signals is the expression of mutated forms of the receptor for each signal that work as dominant negatives. The expression of the dominant negative receptors will block specific pathways activated by the inducer; thus, if that inducer is required for neural crest induction, the expression of neural crest markers should be inhibited. There are also dominant negative constructs for molecules that work within the pathway.

(2) *Gain-of-function experiments.* The putative inducer is added to competent ectoderm, or the pathway is activated; the induction of neural crest markers is then analyzed. The inducer can be added directly to the medium in which the ectoderm is cultured, or it can be added to the embryos. Cells expressing the inducer or beads soaked with the inducer can be grafted in competent ectoderm to test if they are able to induce neural crest. Finally, another important requirement is that an inducer needs to be expressed in the correct place and time. If the putative inducer is never expressed in the tissues that have been identified as the source of neural crest inducer, it is unlikely that it could be the real inducer of the neural crest.

The molecules that have thus far been identified as neural crest inducers are described here.

Bone morphogenetic protein

According to the default model of neural induction, the ectoderm produces BMPs, which inhibit the specification of the ectoderm as neural cells, whereas the organizer or dorsal mesoderm secretes anti-BMPs (Noggin, Chordin, Nodal), which block the neural inhibition and allow the ectodermal cells to adopt a neural fate (DeRobertis and Kuroda, 2004). According to this model (Aybar and Mayor, 2002), the neural crest is specified at the border of the neural plate, where an intermediate level of BMP is reached (Figure 26.4). Thus, the BMP activity in that region is not as high as in the epidermis and not as low as in the neural plate. As predicted by this model, the inhibition of BMP activity in the ectoderm will expand the neural crest domain, because the thresholds that specify these cells are now reached in a wider ectodermal domain (see Figure 26.5). The expression of a dominant negative BMP receptor and expression of the anti-BMP molecules noggin and chordin in amphibian embryos and different BMP mutants in zebrafish exhibits a phenotype with an expanded neural crest domain (Marchant et al., 1998; Nguyen et al.,

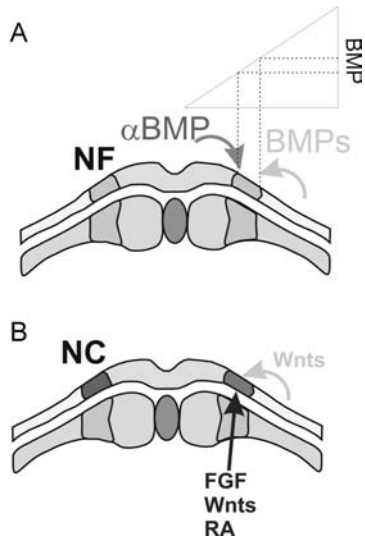

FIGURE 26.4 **Molecules involved in neural crest induction. A,** An initial gradient of bone morphogenetic protein (BMP) activity is generated in the ectoderm, with high levels in the epidermal region, low levels in the neural plate region, and intermediate levels in the neural crest region. This intermediate level of BMP specifies an early stage of neural crest development (*pale purple*). **B,** An additional step that is dependent on Wnts, fibroblast growth factors, and retinoic acid is required to completely specify neural crest cells (*dark blue*). The initial gradient of BMP is generated by BMP produced by the epidermis and anti-BMP molecules produced in the dorsal or medial region of the embryo, whereas the mesoderm produces fibroblast growth factors, Wnts, and retinoic acid. The epidermis produces Wnt signaling. (See color insert.)

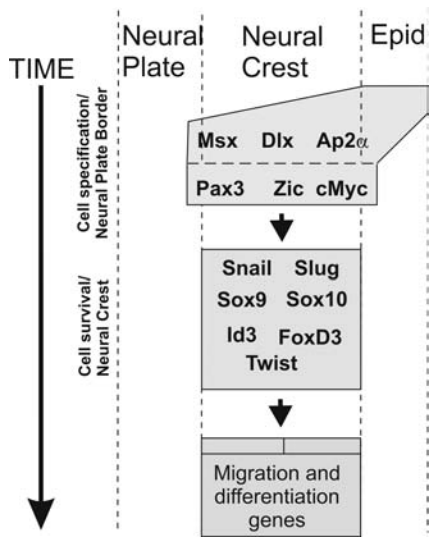

FIGURE 26.5 **Genetic network in the neural crest.** The genes expressed in the neural crest are grouped into three kind of genes: cell specification or neural plate border genes (*top box*), cell survival or neural crest genes (*middle box*), and migration/differentiation genes (*bottom box*). The hierarchical relationship is indicated by the arrow. The two small boxes on the top of the bottom box indicate that, for the last steps of migration and differentiation, many of the genes that were used for earlier steps of neural crest development are also recruited later.

1998). In addition, treatments of competent ectoderm with increasing levels of BMP are able to induce the expression of neural plate, neural folds, and epidermal markers, sequentially (Wilson et al., 1997; Marchant et al., 1998; Neave et al., 1997; Nguyen et al., 1998). The addition of BMPs to dissected neural plate induces the expression of neural crest markers (Liem et al., 1995). Taken together, all of these data support the notion that a specific level of BMP, within a BMP gradient, plays an important role in the specification of the neural plate border. We should point out here that this precise level of BMP is not sufficient to induce neural crest and that additional signals are required to induce the neural crest within the neural plate border.

Fibroblast growth factor

One of these additional signals is FGF. The treatment of competent ectoderm with a combination of BMP inhibitors and FGF is able to trigger the expression of neural crest markers (Mayor et al., 1997; Monsoro-Burq et al., 2003). The expression of FGF into whole embryos leads to an expansion of neural crest markers. The inhibition of FGF activity by the expression of dominant negatives or by treatment with FGF inhibitors completely blocks neural crest induction. Thus, gain- and loss-of-function experiments support the notion that FGF is involved in neural crest induction.

In situ hybridization of *fgf8* demonstrates that it is expressed in the mesoderm underlying the neural crest and that the specific inhibition of FGF8 in the dorsolateral mesoderm abolishes neural crest induction (Monsoro-Burq et al., 2003). In conclusion, one of the neural crest inductive signals produced by the mesoderm corresponds with FGF.

Wnts

The treatment of ectoderm with a precise level of BMP and Wnt also induces the expression of neural crest markers (LaBonne and Bronner-Fraser, 1998; Saint-Jeannet et al., 1997; Lekven et al., 2001; Deardorff et al., 2001; Villanueva et al., 2002; Garcia-Castro et al., 2002; Tribulo et al., 2003; Lewis et al., 2004; Bastidas et al., 2004). The inhibition of Wnt signaling by expressing different Wnt inhibitors or dominant negatives of the Wnt pathway produces a strong inhibition in neural crest induction. Grafts of cells expressing Wnt0001 lead to an upregulation of neural crest markers in the chick (Garcia-Castro et al., 2002).

It is clear that at least two tissues that are adjacent to the prospective neural crest cells express Wnt ligands. The dorsal epidermis in chick expresses Wnt6, and it has been proposed as one of the inductive signals produced by the epidermis. The dorsolateral mesoderm, which has been identified as a source of inductive signals for the neural crest, expresses Wnt0008 in Xenopus, zebrafish, and chick embryos. Thus, there is compelling evidence that several members of the Wnt family work as neural crest inducers.

Retinoic acid

The treatment of amphibian embryos with RA produces the ectopic expression of neural crest markers, and so does the expression of an activated form of the RA receptor (Begemann et al., 2001; Villanueva et al., 2002). The *in vitro* culture of anterior neural folds or neuralized ectoderm, which does not differentiate into neural crest cells, exhibits an upregulation of neural crest

markers after treatment with RA. The expression of dominant negative forms of RA receptors inhibits neural crest induction *in vitro*. Taken together, these experiments indicate that RA is also involved in neural crest specification.

Is RA present at the right time and place to be a neural crest inducer? Because RA is not a protein, it is not possible to analyze the expression of a single gene to know where RA is present in the embryo, and measurements of RA in whole embryos have been difficult. It is possible to have an idea where RA could be active by analyzing the expression of the enzymes involved in its synthesis and degradation. Raldh2 is the key enzyme required for RA synthesis. This enzyme is expressed in posterior and paraxial mesoderm. Thus, this mesoderm, which has inductive activity, could be a source of RA. The anterior region of the embryo, where no neural crest is formed, expresses Cyp26, which is one of the enzymes responsible for the degradation of RA. In conclusion, RA has a neural crest inductive activity, and it is present in some of the tissues in which the inducers are produced.

Notch/delta

Notch is a large, single-transmembrane domain protein that acts as a receptor for the ligand Delta (or Serrate). The ligand is also a transmembrane protein, and, after ligand stimulation, the intracellular domain of Notch is cleaved and transported into the nucleus (see chapter 1 by Soriano and Hosch in this book). In the nucleus, the intracellular domain of Notch interacts with other transcription factors and regulates the expression of target genes. It has been shown that Notch is involved in neural crest induction in zebrafish, chick, and *Xenopus* embryos (Cornell and Eisen, 2000; 2002; Endo et al., 2003; Glavic et al., 2004). The activation of Notch leads to an expansion of the neural crest domain, whereas its inhibition produces a reduced population of neural crest cells. Although there is consensus in the role of Notch during neural crest induction in these three animal models, the specific molecular mechanism as well as the expression of the different components of this pathway seems to be different among them (Cornell and Eisen, 2005).

2. A Model of Neural Crest Specification

The current model for neural crest induction proposes that they are specified at the border of the neural plate in two steps. During the first step, a gradient of BMP activity is established in the ectoderm (see Figure 26.4, A). This gradient is formed as a consequence of the interaction between BMP that is produced in the ventral or lateral ectoderm and BMP-binding molecules produced in the dorsal or medial mesoderm. Within this gradient, high levels of BMP activity specify epidermis; low levels specify neural plate; and intermediate levels are required for early neural fold specification. These early neural folds are not completely induced to become neural crest, because additional signals are required. Additional signals, FGFs, Wnts, and RA work together to transform the early neural fold into neural crest cells. It is not known why three different transforming signals are required and how they interact with each other.

Others signaling molecules, such as Notch/Delta, are also important for neural crest specification, but experiments in Xenopus and chick embryos suggest that they work by controlling the levels of BMP in the neural fold region. Thus, it is very likely that Notch/Delta works downstream of the BMP gradient but that it forms a loop of activity that controls the maintenance of the BMP

levels. It is interesting to note that these three transforming molecules (FGF, Wnts, and RA) are also involved in the posteriorization of the neural plate, so it seems that the posteriorization of the neural plate is very much linked with the specification of the neural crest. It is well known that the neural crest is not formed in the most anterior neural fold, and it has been proposed that these three molecules work by transforming the anterior neural fold, which is induced within the BMP gradient, into a more posterior neural fold (posteriorization), which corresponds with neural crest cells.

3. Genetic Network in the Neural Crest

After the neural crest has been specified by the combination of extracellular signals described previously, a large group of genes is expressed in the neural crest cells. Many of these genes code for transcription factors, and a cascade or network of transcriptional regulation is set up in the neural crest cells. It is not clear which are the first members and which are the last ones of this cascade; however, on the basis of the time of expression, the region of expression, and some functional experiments, a possible regulatory network has been proposed (Figure 26.5). For simplicity, the network has been divided in three groups of genes (Mayor and Aybar, 2001; Muelemans and Bronner-Fraser, 2004; Steventon et al., 2005). The first group, called *cell specification* or *neural plate border specifier genes,* contains the earliest genes that are expressed in the neural plate border. Their expression is not restricted to the prospective neural crest, because they are expressed in a wider domain that includes prospective epidermal and neural plate cells. They control the expression of the second group of genes, called *cell survival* or *neural crest specifier genes*. The expression of this second group of genes is restricted to the prospective neural crest cells, and most of these genes control the survival of the neural crest cells. The third group of genes is the last to be expressed in the neural crest. They are starting to migrate and differentiate, and they are the target of the two previous groups of genes; they have been called *migration/differentiation* or *neural crest effector genes*. They are involved in the last step of neural crest differentiation, and they are usually involved in controlling cell adhesion, cytoskeleton, and cell differentiation.

We will describe briefly some of the genes in each of the first two groups.

Cell specification/neural plate border specifier genes

This group of genes has been divided in two subgroups: early (*Msx, Ap2,* and *Dlx*) and late (*Zic, Pax3,* and *c-Myc*).

The early genes are initially expressed in the nonneural ectoderm, and they are later restricted to the neural fold region in Xenopus and chick embryos. Their expression pattern correlates with the assumed ventral–dorsal gradient of BMP activity, and the identification of cis-regulatory elements in some of these genes indicates that they are very likely to be direct targets of BMP. Loss-of-function experiments in mouse, zebrafish, and Xenopus embryos show that they are required for the early specification of the neural crest.

The late genes are expressed in a more restricted domain than the early genes. They are not expressed in the entire epidermis during earlier stages, but they are present in a wider domain than the premigratory neural crest cells. Loss- and gain-of-function experiments have shown that they play a fundamental role in neural crest specification.

Cell survival/neural crest specifier genes

These genes are expressed later than the cell specification genes, and they are only expressed in the neural crest territory. They encode for transcription factors of the *Snail* and *SoxE* family of genes and for the genes *FoxD3, Id3,* and *Twist*. It is also important to note that *Snail, Slug,* and *FoxD3* all function as transcriptional repressors; this means that there are additional transcription factors that mediate the activity of these genes. Gain- and loss-of-function studies for each of these genes show that they are essential for neural crest development and that they probably control different aspects of neural crest specification, migration, and/or differentiation. In addition, many of these genes are involved in cell survival.

III. REGIONALIZATION OF NEURAL CREST CELLS

After the neural crest cells are induced, they start to migrate, and they differentiate into a wide variety of neural crest derivatives (see Figure 26.2). According to their final positions after migration, the neural crest cells are able to differentiate into cartilage, bone, connective tissue, neurons, glia, muscle, pigment cells, and adrenal cells. This huge range of different kinds of cells seems to be determined by a combination of intrinsic factors that are specified in the genetic network activated during neural crest induction, and by external cues coming from the environment that the cells encounter during their migration or after they have reached their final target.

The neural crest can be divided into four different domains according to the anterior–posterior axis and the derivatives that they originate: cephalic, trunk, vagal, and cardiac.

A. Cephalic Neural Cells

The cranial or cephalic neural crest cells originate from the massive cephalic neural folds, and they migrate dorsolaterally to generate the craniofacial mesenchyme, which will differentiate into the cartilage, bone, cranial neurons, glia, and connective tissue of the face.

The neural crest from rhombomeres 1 and 2 will migrate to the mandibular arch and form the jaw and middle ear. The same neural crest population will form the frontonasal process, which will generate the bones of the face. The neural crest cells from rhombomere 4 will migrate into the second arch and form the hyoid cartilage of the neck and the stapes bones of the middle ear. Neural crest from rhombomere 6 will populate the third and fourth arches to form the thymus, parathyroid, and thyroid glands. Neural crest from rhombomeres 3 and 5 does not migrate into the surrounding mesoderm; instead, it migrates in the streams of adjacent rhombomeres.

B. Trunk Neural Crest

Trunk neural crest cells originate from the posterior part of the embryo from the equivalent to somite 7 to the most posterior end of the embryo (see Figure 26.2). They follow two main routes of migration at two different times. The first wave of migration takes a ventrolateral pathway through the somites (the anterior half of each somite in the chick and the middle of each somite in zebrafish).

Some neural crest cells will remain in the somite and form the dorsal root ganglia, which contains sensory neurons. Other cells will migrate more ventrally, and they will form the sympathetic ganglia, the adrenal medulla, and the nerves around the aorta.

A second wave of neural crest cells follows a dorsolateral pathway under the ectoderm. They will become pigment-synthesizing melanocytes that will move though the skin toward the ventral midline of the belly.

For the neural crest cells to migrate, they undergo an epithelial-to-mesenchymal transformation during which the cells need to lose their attachment to other cells, become motile, and move away from the epithelial sheet. Intracellular factors that control the cytoskeleton, such as RhoB, are required for neural crest migration together with the downregulation of molecules involved in cell adhesion and attachment, such as cadherins and tight junction proteins. The route taken by migrating neural crest cells is controlled by a combination of permissive and restrictive signals. Extracellular matrix molecules (e.g., fibronectin, laminin, tenascin, collagens, proteoglycans) promote migration. The neural crest cells express integrins that interact with some of these extracellular matrix proteins, thereby controlling migration and the survival of the neural crest cells.

Important factors for patterning the migratory neural crest pathways are the negative signals that impede migration in the regions in which they are expressed. Some of these negative signals are ephrins (and Eph receptors), Slit (and Robo receptors), and semaphorins (and neuropilin receptors). These proteins are expressed in the regions in which the neural crest cells do not migrate, whereas their receptors are expressed by the neural crest cells (Krull et al., 1997; Smith et al., 1997; Gammill et al., 2006; Santiago and Erickson, 2002; De Bellard et al., 2003). Loss-of-function experiments involving these factors or receptors lead to abnormal neural crest migration into regions in which the neural crests cells are normally absent. Interestingly, similar molecular interactions have been found for the movement of axonal growth cones.

C. Vagal and Cardiac Neural Crest

The vagal neural crest will form the parasympathetic (enteric) ganglia of the gut, and the cardiac neural crest will produce the entire muscular–connective tissue wall of the large arteries as they arise from the heart as well as the septum, which separates the pulmonary circulation of the aorta. The neural crest located at the level of the first three somites in chicks gives rise to vagal and cardiac crest, but vagal crest cells also originate from more posterior regions, like the crest located at somites 4 through 7 and posterior to somite 28 (see Figure 26.2).

IV. HUMAN PATHOLOGIES

Several human pathologies are produced by a failure of neural crest development. The defect can occur during the early specification of the neural crest as part of its migration or differentiation. One of the abnormalities associated with the cephalic neural crest is DiGeorge syndrome. Patients with this syndrome have an abnormal migration of the neural crest in the pharyngeal arches. They have hypocalcemia that arises from defects in the parathyroid glands, thymic hypoplasia, and outflow tract defects of the heart. The facial anomalies include

low-set ears, a small mouth and philtrum (the space between the upper lip and the nose), and cleft palates. DiGeorge syndrome is associated with deletions on the long arm of chromosome 22, and at least two genes present in that region are involved in this syndrome: *Tbx1* and *Tuple1* (Baldini, 2005).

A failure of vagal neural crest cell migration to the colon results in the absence of enteric ganglia and, thus, in the absence of peristaltic movement in the bowel. The human pathology associated with this failure is called Hirschsprung disease (or megacolon), and it has been demonstrated that the gene *Sox10* plays a key role in the generation of this pathology (Farlie et al., 2004).

Several animal models have been developed that reproduce some or all aspects of these and other neural crest pathologies. The most frequent organism used to study these pathologies is mouse, because it is a mammal; however, organisms such as Xenopus and zebrafish have also been very useful for the study of the role of specific genes on a particular phenotype that mimics the human syndrome. This is a very active area of research that is growing very quickly. Understanding the normal cellular/molecular/genetics mechanisms of neural crest development in different animal models will be essential for the further understanding of human syndromes.

SUMMARY

- The neural crest is induced at the border of the neural plate by signals that come from the epidermis, the neural plate, and the underlying mesoderm.
- Two kinds of signals are required to induce neural crest: an intermediate level of BMP activity and a second signal.
- The second neural crest inductive signal involves Wnt, FGF, or RA.
- After the neural crest is induced, a network of transcription factors is activated, first in the epidermis, then in a wider region of the neural plate border, and finally in the neural crest.
- Anterior and posterior neural crest gives rise to very different kinds of cells, tissues, and organs.
- Failure during neural crest development is associated with several human pathologies.

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GLOSSARY OF TERMS

Determination
An irreversible decision made by an undifferentiated cell that restricts its differentiation.

Ectoderm
One of the three germ layers of the embryo. It gives rise to the epidermis, the neural plate, and the neural crest.

Embryonic induction
The interaction between one inducing tissue and another responding tissue. As a result, the responding tissue undergoes a change in fate.

Neural crest
The transient tissue found in vertebrate embryos that is formed at the border of the neural plate and that undergoes a transformation into migrating cells, which differentiate into a huge variety of cells. It gives rise to most of the head, the peripheral nervous system, and the pigments of the skin, among other things.

Neurulation
The developmental process in vertebrate embryos that results in the formation of the neural plate and that terminates with its closure into the neural tube.

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27 DETERMINATION OF PREPLACODAL ECTODERM AND SENSORY PLACODES

SALLY A. MOODY

Department of Anatomy and Cell Biology, The George Washington University, Washington, DC

INTRODUCTION

The vertebrate head contains a number of specialized sensory organs that arise from embryonic ectodermal thickenings called the *cranial sensory placodes* (von Kupffer, 1891; reviewed by Webb and Noden, 1993; Baker and Bonner-Fraser, 2001; Streit, 2004; Brugmann and Moody, 2005; Schlosser, 2005; 2006). During gastrulation, the ectoderm surrounding the anterior neural plate becomes specified to form peripheral sensory structures, a region that is called the *lateral neurogenic zone* (Figure 27.1). The more medial region of this zone, which includes the edge of the neural plate, gives rise to the neural crest, and the more lateral region gives rise to a preplacodal ectoderm (PPE), which later separates into individual cranial sensory placodes (Knouff, 1935; LeDouarin et al., 1986; reviewed by Schlosser and Northcutt, 2000). The cranial sensory placodes are distinct from other ectodermal thickenings (also called *placodes*) that form in the nonneural epidermis to give rise to the teeth, hair follicles, and feathers (Pispa and Thesleff, 2003; see Chapter 28). During neurulation, signals from underlying tissues cause the PPE to separate into many discrete placodes, which are histologically recognized as patches of thickened ectoderm and which have distinct developmental fates (Figures 27.1 and 27.2). These placodes will then produce both the structural and neural elements of numerous cranial secretory tissues and sensory organs, including the anterior pituitary gland, the olfactory epithelium, the lens, and the auditory and vestibular organs. In addition, cranial nerve sensory ganglia contain cells derived from both the placodes and the neural crest (see Chapter 26). Thus, the PPE gives rise to many important structures in the vertebrate head. However, although the cranial placodes have been histologically recognized

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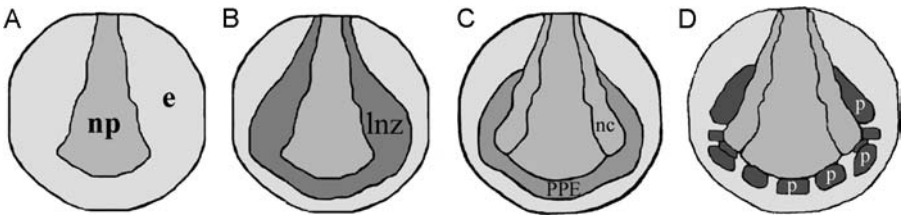

FIGURE 27.1 **The ectodermal domains of the Xenopus embryo at different developmental stages. A,** At gastrulation, the early embryonic ectoderm is divided into the neural plate *(np)* and epidermis *(e)* domains. **B,** Interactions between these two domains establish a border region called *the lateral neurogenic zone (lnz)*, which will give rise to **C,** the medial neural crest *(nc)* and the lateral preplacodal ectoderm *(PPE)*. **D,** Subsequently, the preplacodal ectoderm breaks up into the individual placodes *(p)*. (See color insert.)

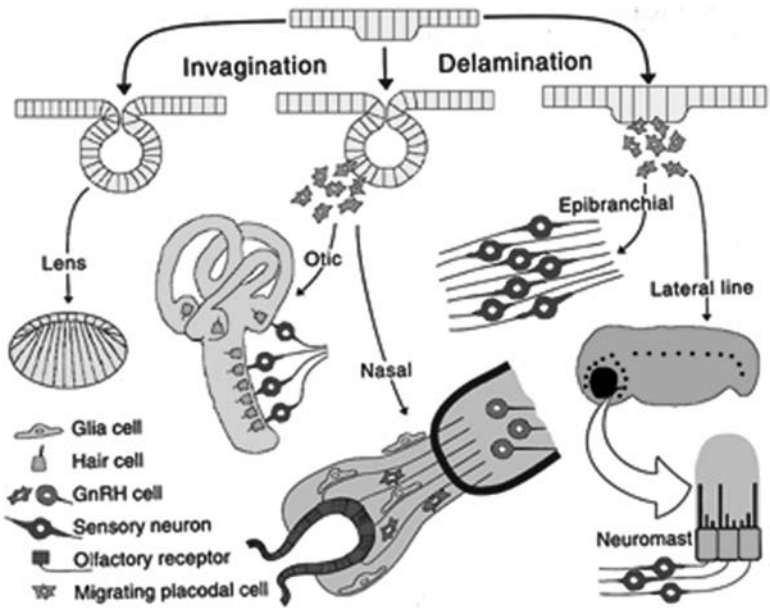

FIGURE 27.2 **Placodes give rise to several sensory derivatives.** The initial epithelial thickening can invaginate to give rise to a pit (olfactory) or a vesicle (hypophyseal, lens, otic), or cells can simply delaminate and migrate to a secondary position (cranial ganglia, lateral line). Note the numerous cell types that derive from the cranial placodes. (Modified from Webb and Noden, 1993 and from Brugmann and Moody, 2005.)

for more than a century, very little is known about the molecular mechanisms that specify the development of these important sensory precursors. The recent identification of genes that are highly expressed in the PPE and in individual placodes has allowed researchers to begin to reveal the molecular pathways that induce and specify the fate of these important embryonic cells.

I. CRANIAL SENSORY PLACODES GIVE RISE TO DIVERSE STRUCTURES

Structurally, much is known about the development of the various cranial sensory placodes. This development is characterized by extensive morphogenetic

rearrangements. Some placodes (i.e., hypophyseal, olfactory, lens, otic) invaginate as cup-shaped structures (see Figure 27.2). The olfactory cup, which is also called the *olfactory* or *nasal pit,* further folds to line the nasal cavity as a sensory epithelium. The hypophyseal, lens, and otic cups, however, continue to invaginate, and they eventually pinch off from the surface ectoderm to form epithelial-lined vesicles that then further differentiate into highly specialized structures. In other placodes, the underlying basement membrane of the thickened epithelium becomes fragmented, thereby allowing cells to delaminate from the epithelium. Some migrate within the ectoderm to form patches of sensory organs (e.g., the lateral lines), and some migrate away from the surface ectoderm to coalesce within the head mesenchyme as sensory ganglia.

In each placode, the cells can adopt a variety of cell fates, including secretory cells, sensory receptor cells, neurons, glia, or supporting cells, depending on their placode of origin (see Figure 27.2). The hypophyseal placode (also called *adenohypophyseal*) first lies on the midline surface of the embryo, and it later occupies the dorsal midline of the oral ectoderm as the stomodeum forms. It then invaginates to form Rathke's pouch, and it pinches off as a vesicle to form the adenohypophysis (anterior pituitary gland), the cells of which secrete a number of peptide hormones. The olfactory placode (also called *nasal*) gives rise to the primary olfactory receptor neurons that detect odorants, glia, mucus-secreting, and structural cells of the olfactory epithelium; in some animals, it also gives rise to the related vomeronasal epithelium, which detects pheromones. In addition, some cells migrate from the olfactory placode into the hypothalamus to become secretory components of the gonadotropin-releasing hormone system, and some coalesce into a small ganglion that is associated with the terminal nerve. The lens placode gives rise to the lens vesicle in the anterior segment of the eye, which contains the crystalline-secreting cells that focus light on the neural retina. Two placodes contribute neurons to the trigeminal ganglion: the ophthalmic placode (also called *profundal*), which is located dorsal to the eye, and the trigeminal placode (also called *Gasserian* or *maxillomandibular*), which is located just caudal to the eye. These cells are equivalent to dorsal root ganglion cells, and they provide the sensory innervation of the face, the oral cavity, and the scalp. The otic placode gives rise to both the auditory and vestibular parts of the entire inner ear, including the mechanosensory hair cells, the supporting cells, the endolymph secreting cells, the biomineralized otoliths, and the vestibuloacoustic ganglia (see Chapter 29). A series of epibranchial placodes forms in the branchial arch ectoderm between adjacent endodermal pharyngeal pouches ventral to the otic placode. Cells from the epibranchial placodes migrate into the branchial mesenchyme to become neurons in the distal sensory ganglia of three cranial nerves. Those of the facial nerve (called the *geniculate ganglion*) innervate the taste buds; those of the glossopharyngeal nerve (called the *petrosal ganglion*) innervate the taste buds, the heart, and the visceral organs; and those of the vagus nerve (called the *nodose ganglion*) innervate the heart and other visceral organs.

All vertebrates have these placodes in common, but there are numerous species variations (reviewed by Baker and Bronner-Fraser, 2001; Schlosser, 2005; 2006). For example, in some animals, the derivatives of the profundal and trigeminal placodes are maintained as separate sensory ganglia, and, in some, there are up to six separate epibranchial placodes with associated ganglia. In amphibians and fish, there is an additional lateral line sensory

system that is specialized for aquatic life. This system consists of islands of sensory organs (receptor and supporting cells as well as the sensory neurons that innervate them) that have striking similarities to inner ear receptor organs and that are distributed across the head and trunk epidermis. Mechanoreceptive neuromast organs detect water turbulence, and electroreceptive ampullary organs detect electrical fields. Lateral line cells derive from a dorsolateral placode located adjacent to the otic placode. In some amphibians, there are also hypobranchial placodes that are located ventral to the second and third pharyngeal pouches that give rise to the hypobranchial ganglia, the function of which is presently unknown (Schlosser, 2003). We do not yet understand the evolutionary mechanisms that have given rise to the diversity of these structures across species (reviewed by Schlosser, 2005; see Chapter 6), but, as is discussed in the later sections of this chapter, many of the genes involved in placode development are highly conserved from invertebrates to vertebrates.

II. INITIAL FORMATION OF THE PREPLACODAL ECTODERM

The classic descriptions of cranial sensory placode formation proposed that all of these distinct structures derive from a common precursor region called the PPE, which forms around the anterior margin of the neural plate (see Figure 27.1). Although there are data that suggest that each placode may be individually induced and specified during development (Graham and Begbie, 2000; Begbie and Graham, 2001), fate mapping and gene expression studies strongly argue that the cranial sensory placodes derive from a common precursor region that is distinct from the neighboring ectodermal fields and that this region is initially molecularly biased toward a general placodal fate (reviewed by Streit, 2004; Ahrens and Schlosser, 2005; Schlosser, 2005; 2006). Because the neural crest and the placodes are both derived from the lateral ectoderm that surrounds the neural plate, and because both tissues contribute to the peripheral nervous system, it has been suggested that the placodes might be induced by mechanisms similar to those that induce the neural crest (see Chapter 26). However, there are several reasons that there also should be distinct differences. The PPE forms lateral to the neural crest and extends around the most rostral tip of the neural plate, whereas neural crest is absent from this region; placodes form only in the head, whereas neural crest cells extend to the caudal regions of the trunk (see Figure 27.1). It has only been in recent years, as a consequence of the cloning and of the characterization of several genes that are highly expressed in the PPE and early placodes, that it has been possible to experimentally examine the mechanisms that induce the PPE and to determine how these compare with those that induce neural crest.

A. The Role of Neural Plate/Nonneural Ectoderm Signaling

Several studies in many animal models have demonstrated that the formation of a lateral border zone that gives rise to the neural crest requires an interaction between the neural plate and the nonneural ectoderm as the early neural plate forms (reviewed by Meulemans and Bronner-Fraser, 2004; see Chapter 26). These interactions appear to initiate the expression of transcription factors

(e.g., *dlx3, msx1 pax3, zic*) that have the following characteristics: (1) they are typical of the lateral border zone; (2) they are necessary for the endogenous expression of "neural crest-specifying" genes; and (3) they cause the ectopic induction of several neural crest markers at the margin of a piece of neural plate grafted into a nonneural ectodermal domain. Evidence of a similar interaction to initiate the development of the PPE is now accumulating. A large body of literature demonstrates that signals from the neural plate are required for the induction of individual placodal structures (reviewed by Baker and Bronner-Fraser, 2001). These studies mostly analyzed late stages using placode-specific markers or morphology to indicate the induction of the tissue of interest (e.g., the otic vesicle, which can be easily identified by histology) and thus do not directly address whether this interaction is necessary for the induction of the panplacodal fate of the PPE. However, recent studies have taken advantage of newly described genes expressed in the early PPE (e.g., *six1, eya1, Xiro1*) to show that neural plate grafts placed into nonneural ectoderm also induce PPE (Woda et al., 2003; Glavic et al., 2004; Ahrens and Schlosser, 2005). Thus, the interaction between the newly formed neural plate and the adjacent nonneural ectoderm specifies a lateral neurogenic zone that commonly gives rise to both the neural crest and the PPE (Figures 27.1 and 27.3). What has not been clear from these studies is whether the interaction specifies two separate domains (neural crest and PPE) or a single, presensory zone that later separates into two fields.

The latter idea is supported by recent studies that indicate that transcription factors expressed in the nonneural ectoderm are required for both neural crest and PPE formation. One factor, *foxi1*, is a member of the Drosophila *forkhead* family. During gastrulation, it is expressed throughout the anterior–

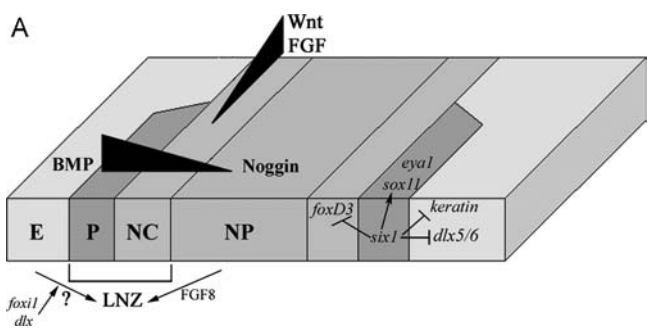

FIGURE 27.3 Several steps are involved in forming the preplacodal ectoderm (PPE). The embryonic ectoderm has been flattened into a sheet and into the four major domains illustrated. *E,* Epidermis; *P,* PPE; *NC,* neural crest; *NP,* neural plate. Inductive interactions are depicted on the left side. First, interactions between the neural plate and the epidermis *(bottom of figure)* cause a lateral neurogenic zone *(LNZ)* to form; this will further divide into neural crest and preplacodal ectoderm. Fibroblast growth factor (FGF)8 is likely one of the responsible signals from the neural plate. The *foxi1* and *dlx* genes are likely to regulate yet-to-be identified signaling factors from the epidermis. In addition, the four ectodermal domains are specified in response to a gradient of bone morphogenetic protein (BMP) signaling, which is antagonized by anti-BMP factors from the midline, such as Noggin. Finally, a gradient of posteriorizing signals (Wnt, FGF) is required for neural crest formation and for the inhibition of PPE formation. On the right side are some of the genes that are expressed in the neural crest, in the PPE, and in the epidermis. Experiments show that *six1* promotes the expression of placode genes *(sox11, eya1)* at the expense of neural crest *(foxD3)* and epidermal *(keratin, dlx)* genes.

ventral embryonic ectoderm, and later it is expressed in a U-shaped domain that surrounds the anterior neural plate (Matsuo-Takasaki et al., 2005). At first, its expression domain extends to the border of the *sox2*-expressing neural plate ectoderm, but later it recedes from the lateral neurogenic zone surrounding the anterior neural plate. In ectodermal (animal cap) explants, *foxi1* expression is induced by bone morphogenetic protein (BMP) and repressed by Chordin, and, in whole embryos, its expression domain is expanded by BMP mRNA injection; this is typical of epidermal genes (see Chapter 12). The knockdown of *foxi1* expression by the injection of antisense morpholino oligonucleotides (MOs) expands the *sox2*-expressing neural plate domain, but MO also represses the expression of both neural crest *(foxD3)* and placodal *(six1, eya1)* genes, which indicates that its early expression at the neural/nonneural border is required for both derivatives of the lateral neurogenic ectoderm. Similar results have been reported for three *dlx* genes *(dlx3, dlx5, dlx6)*, which are related to Drosophila *distal-less*. They are initially expressed throughout the nonneural ectoderm, and they are induced by BMPs (Luo et al., 2001a; 2001b; Woda et al., 2003). In Xenopus, the initial expression boundaries of *dlx5* and *dlx6* abut the neural plate, whereas the expression boundary of *dlx3* abuts the lateral neurogenic zone. Gain-of-function studies in frog indicate that these genes repress neural plate genes and that they are required for the expression of both neural crest and placode markers during gastrulation stages (Feledy et al., 1999; Beanan and Sargent, 2000; Luo et al., 2001a; Woda et al., 2003). In the chick, *dlx5* expression also represses neural plate markers and promotes neural fold markers (McLarren et al., 2003). These experiments suggest that *dlx* genes promote the formation of the lateral neurogenic border zone. This was confirmed by experiments in which neural plate tissue was grafted into nonneural ectoderm (Woda et al., 2003). Both neural crest and PPE markers were induced when grafts were placed in areas that expressed *dlx* genes, but, when grafts were placed in a region where the activity of all *dlx* genes was downregulated by the expression of a pan–*dlx*-repressor construct, neither marker was induced. The common effects on both neural crest and PPE genes and the timing of the effects reported in these studies indicate that both *foxi1* and *dlx* genes have an early function in subdividing the embryonic ectoderm into neural versus nonneural domains, and in establishing a lateral region in the ectoderm surrounding the neural plate that can give rise to both the neural crest and PPE (see Figure 27.3).

The nature of the signal(s) between the neural plate and the nonneural ectoderm that mediate the formation of the lateral neurogenic zone is still uncertain. However, several experiments implicate members of the fibroblast growth factor (FGF) family. Older studies that focused on individual placode markers or morphology showed a role for FGF3 and FGF8 in a variety of embryos (reviewed by Baker and Bronner-Fraser, 2001). More recent studies in Xenopus and chick using PPE molecular markers showed that FGF8, which is expressed in the anterior neural plate, is involved (Ahrens and Schlosser, 2005; Litsiou et al., 2005). Experimental manipulations that increased FGF8 levels in nonneural ectoderm induced PPE markers, and a reduction of FGF signaling either with a general FGF receptor inhibitor or with FGF8-specific MOs repressed PPE markers. However, studies in zebrafish show that, although FGF8 and FGF3 are required for otic placode induction, they are not required for the expression of some PPE marker genes (Phillips et al., 2001; Maroon et al., 2002; Leger and Brand, 2002; Liu et al., 2003; Solomon

et al., 2004). Therefore, it remains to be determined whether all vertebrates share a common PPE induction by a neural plate source of FGF8.

Interestingly, the studies previously mentioned showed that FGF8 alone cannot induce PPE marker genes. The effectiveness of FGF8 for inducing PPE genes depended on the concomitant reduction of the level of BMP signaling in the nonneural ectoderm (Ahrens and Schlosser, 2005). For example, although beads coated with FGF8 implanted in the nonneural ectoderm could induce low levels of PPE gene expression, combining FGF8 with Noggin caused a dramatic induction. The same results were found after grafting animal cap ectoderm that expressed either FGF8 alone or in combination with Noggin. Thus, in addition to FGF signaling, PPE induction also appears to require reduced BMP signaling.

B. The Role of Bone Morphogenetic Protein Signaling

The vertebrate central nervous system forms in the embryonic ectoderm largely as a consequence of the dorsal expression of several molecules that antagonize the signaling of BMPs, which are highly expressed in ventral ectoderm (see Chapter 12). Several studies indicate that, although high concentrations of BMP antagonists such as Noggin and Chordin induce neural plate formation, intermediate concentrations induce neural crest formation (see Chapter 26). These results led to the idea that a concentration gradient of BMP patterns the embryonic ectoderm into several subdomains, with epidermis forming at the high end of the gradient, neural plate forming at the low end of the gradient, and neural crest forming at intermediate levels (see Figure 27.3). This gradient may be established by the expression of BMP antagonists in the dorsal midline mesoderm that diffuse laterally through both the mesoderm and the adjacent ectoderm or the local expression of antagonists in the underlying tissues.

Because the PPE develops between the neural crest and the epidermis, it was proposed that it is also likely to form at an intermediate—and perhaps even lower—level of BMP signaling (Baker and Bronner-Fraser, 2001). It became possible to experimentally test this hypothesis when molecular markers of the PPE *(six, eya)* became available (Esteve and Bovolenta, 1999; Kobayashi et al., 2000; Pandur and Moody, 2000; David et al., 2001; Ghanbari et al., 2001; Bessarab et al., 2004). First, the injection of mRNAs encoding BMP antagonists Noggin and Cerberus induced *six1* expression in Xenopus animal cap explants (Brugmann et al., 2004), which demonstrated that BMP signaling needs to be reduced in the embryonic ectoderm for PPE gene expression. When explants were cultured in different concentrations of Noggin protein, *six1* and *eya1* were highly expressed at very low concentrations (1–5 ng/mL), and their expression was dramatically reduced as Noggin concentration increased to intermediate levels that induced a neural crest gene *(foxD3)* or to high levels that induced a neural plate gene *(sox2)*. These studies indicate that genes that are characteristic of the three early neurogenic fields (i. e., neural plate, neural crest, and PPE) are most strongly induced in ectodermal explants at different concentrations of BMP antagonist. However, two observations are not concordant with a gradient model: (1) ventral nonneural ectoderm transplanted into the neural plate, which is a locale of presumably high BMP antagonist expression, strongly expresses *six1* (Ahrens and Schlosser, 2005); and (2) BMP4 mRNA levels are relatively high along the neural plate

border (Hemmati-Brivanlou and Thomsen, 1995; Streit and Stern, 1999). In none of these studies has the level of BMP protein in the areas of PPE gene expression been measured, so the issue requires further testing. Nonetheless, experiments in whole embryos confirm that PPE gene expression requires a reduction in the level of BMP signaling. The endogenous expression domains of PPE marker genes are reduced when BMP4 is expressed in the lateral neurogenic zone, and they are expanded when BMP signaling is reduced in that zone by the expression of either a dominant-negative BMP receptor or BMP antagonists (Brugmann et al., 2004; Glavic et al., 2004; Ahrens and Schlosser, 2005).

C. The Role of Anterior–Posterior Axis Signaling

Although *six1* and *eya1* can be induced in animal cap explants simply in response to the appropriate concentration of BMP antagonist, this does not occur in the intact embryo. The ectopic expression of Noggin or Chordin in nonneural ectoderm in either chick or frog does not induce the ectopic expression of placode markers (Brugmann et al., 2004; Glavic et al., 2004; Ahrens and Schlosser, 2005; Litsiou et al., 2005). However, there is one exception: if a secondary axis with an endogenous anterior–posterior (AP) axis was induced by ectopically expressed Noggin or Chordin or by grafting the Organizer, *six1* was expressed only at the anterior pole of the ectopic AP axis (Brugmann et al., 2004; Ahrens and Schlosser, 2005). These results indicate that PPE induction is linked to the formation of the AP axis.

It had previously been demonstrated that induction of the neural crest, which extends from midbrain levels to the caudal end of the spinal cord, requires signaling pathways that establish the posterior axis of the neural plate (FGF, Wnt, and retinoic acid; see Chapter 26). Therefore, perhaps the PPE and the placodes, which are confined to the head, are negatively regulated by these posterior signaling molecules. In both animal cap explants and whole embryos, it was demonstrated that the repression of either Wnt or FGF signaling expanded the *six1* expression domain, whereas the activation of Wnt or FGF pathways repressed it (Brugmann et al., 2004). Similarly, the late expression of *foxi1* in the U-shaped domain surrounding the neural plate was also expanded by Wnt antagonists (Matsuo-Takasaki et al., 2005). Experiments in the chick confirmed that combined signaling is required for PPE induction; the expression of *six4* and *eya2* require the reduction of both BMP and Wnt signaling (Litsiou et al., 2005). Finally, it is interesting to note the following: (1) the dorsal endomesoderm that is required for PPE induction in Xenopus is a source of Cerberus (Ahrens and Schlosser, 2005), a secreted protein that inhibits BMP, Wnt, and Nodal signaling and that is necessary for the formation of the head (Piccolo et al., 1999); and (2) the anterior neural plate is also a source of Wnt inhibitors (Bradley et al., 2000; Pera and De Robertis, 2000). Together, these studies indicate that, although neural crest induction requires posteriorizing signals, the PPE only develops in the absence of these signals. In fact, the differential response of neural crest and PPE to posteriorizing factors provides a simple explanation of why neural crest does not form in the most anterior tip of the head and of why sensory placodes do not form in the trunk (see Figure 27.3).

Taken together, these studies suggest that interactions between the neural plate and nonneural ectoderm define a new neurogenic region called the *lateral neurogenic zone;* this term is used to distinguish it from the *border zone,* a term

that is sometimes used to refer to the lateral border of the neural plate, which gives rise to the neural crest. It is not clear whether this lateral neurogenic zone is initially competent to give rise to both neural crest and placodal derivatives and then becomes divided into two separately specified domains or whether the two tissues are distinct from the onset. In support of the first idea are the following observations: (1) the earliest known genes expressed in the lateral neurogenic ectoderm (*foxi1* and *dlx* genes) affect both neural crest and PPE markers in a similar manner; (2) although *six1* and *eya1* are highly expressed at Noggin concentrations lower than those required for a neural crest gene, there is significant overlap in the dose–response curves for the two sets of marker genes (Brugmann et al., 2004); (3) single-cell–mapping studies demonstrated that cells fated to give rise to otic placode are intermingled with rather than separate from future neural crest precursors (Streit, 2002); and (4) the expression domains of several neural crest and placodal marker genes partially overlap (McLarren et al., 2003; Glavic et al., 2004; Schlosser and Ahrens, 2004). In support of the second idea are the following observations: (1) in explant studies, the specification and loss of ectodermal competence for placode markers occur much later than they do for neural crest markers; and (2) in neural plate grafting experiments, placode markers are induced in the surrounding nonneural ectoderm of the host, whereas neural crest markers are induced primarily in the lateral edge of the graft (Ahrens and Schlosser, 2005). Further study of the molecular genetic mechanisms that dictate neural crest versus PPE/placodal fate will be necessary to resolve this issue.

III. GENES THAT SPECIFY PREPLACODAL ECTODERM FATE

Work performed during the past decade has described the expression of a large number of transcription factors in the various placodes of many different animals (reviewed by Baker and Bronner-Fraser, 2001; Schlosser and Ahrens, 2004; Schlosser, 2005; 2006). However, only a few of these genes are expressed throughout the entire PPE from the outset of its formation. In particular, members of two gene families *(six* and *eya)* are candidates for specifying the early preplacodal state, because they are expressed in the characteristic horseshoe-shaped domain that surrounds the anterior neural plate, which corresponds with the morphologic description of the PPE in the early embryo (see Figure 27.1; Esteve and Bovolenta, 1999; Kobayashi et al., 2000; Pandur and Moody, 2000; David et al., 2001; Ghanbari et al., 2001; reviewed by Schlosser and Ahrens, 2004). Recent functional studies indicate that these two families are necessary for the specification of the PPE and its derivatives.

A. *six* Genes

Vertebrate *six* genes are highly related to Drosophila *sine oculis* (*so*). Although *so* is essential for fly visual system formation (Cheyette et al., 1994; Serikaku and O'Tousa, 1994), vertebrate *six* genes play major roles in eye, muscle, kidney, and craniofacial development (Kawakami et al., 1996; Brodbeck and Englert, 2004). All *six/so* proteins contain a highly conserved *six*-type homeodomain, which binds DNA, and an adjacent *six* domain (SD), which appears to increase DNA binding specificity by interacting with cofactors (Pignoni et al., 1997; Kawakami et al., 2000; Kobayashi et al., 2001). Vertebrate *six* genes have been grouped into three subfamilies *(six1/six2; six3/six4; six5/six6)* on

the basis of sequence variations in both the homeodomain and the SD regions (Kawakami et al., 2000). *six1* and *six2* are most closely related to the fly *so,* but neither is known to play a major role in eye development; rather, *six3* and *six6* are critical for vertebrate eye development (see Chapter 25).

Three *six* genes *(six1, six2, six4)* are expressed in vertebrate PPE, placodes, and/or placode derivatives. However, the expression patterns across vertebrates are not identical (reviewed by Brugmann and Moody, 2005). It is not clear whether the differences are the result of true species variation or whether incomplete descriptions from diverse experimental techniques and different developmental stages make the patterns appear disparate. In general, *six1* and *six2* are expressed in the PPE, the placodes (except lens, which expresses *six3*), the lateral line organs, the muscle precursors, the kidneys, the genitalia, and the limb buds. *six4* is typically expressed in the PPE, the placodes, the muscle precursors, the kidneys, the brain, and the eye. It will be very important to fully describe the developmental expression patterns of these *six* genes across all of the animal models and humans to fully understand their roles in placode development and congenital syndromes.

Several experiments indicate that *six1* has a central role in PPE/placode development. First, several loss-of-function studies indicate that *six1* is a required gene. In humans, *six1* mutations lead to some cases of branchio-otic (BO) and branchio-oto-renal (BOR) syndromes, which are autosomal-dominant developmental disorders that are characterized by craniofacial defects and hearing loss (BO, BOR) and by additional malformations of the kidney and the urinary tract (BOR; Ruf et al., 2004). Likewise, *six1*-null mutant mice exhibit severe defects in the development of the nose, the thymus, the skeletal muscles, and the kidneys; in addition, all components of the inner ear fail to form as a result of increased cell death and reduced proliferation in the otic epithelium (Oliver et al., 1995; Laclef et al., 2003; Zheng et al., 2003; Ozaki et al., 2004). Consistent with these mammalian mutations is that the knockdown of *six1* via MO injection in Xenopus embryos results in the loss of early PPE marker gene expression and the expansion of adjacent epidermal *(keratin)* and neural crest *(foxD3)* markers (Brugmann et al., 2004). Second, the increased expression of wild-type *six1* by mRNA injection into the precursors of the lateral neurogenic zone in Xenopus embryos expands the expression domains of other early PPE genes *(sox11* and *eya1)*, and it represses the adjacent epidermal and neural crest domains. These results demonstrate that elevated *six1* expression in the lateral neurogenic zone promotes PPE gene expression at the expense of epidermal and neural crest genes (Figures 27.3 and 27.4).

The functional roles of *six2* and *six4,* which are also expressed in the PPE, have yet to be described in any detail, although they are frequently used as placode marker genes in a number of animal models. To our knowledge, no human syndromes have been assigned to mutations in *six2* or *six4,* and phenotypes of *six2* null mice have not yet been reported. *six4* null mice do not have obvious craniofacial defects or hearing loss (Ozaki et al., 2001), but this lack of phenotype may be the result of redundant functions between the family members. For example, a recent report indicates that double *six1/six4* null mutant mice have more severe defects than either of the single mutants (Grifone et al., 2005), although the placode defects were not characterized. However, because *six1, six2,* and *six4* have distinct roles in myogenic differentiation and in kidney development (Ohto et al., 1998; Spitz et al.,

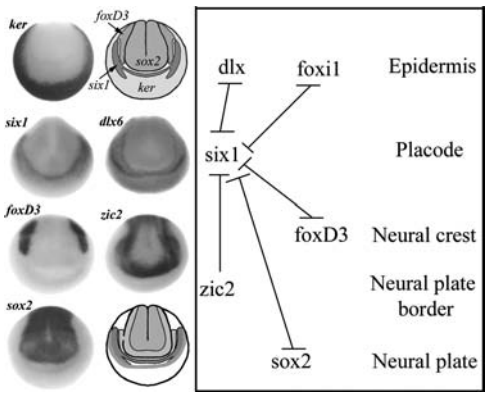

FIGURE 27.4 Boundaries between the four ectodermal domains may be formed by mutually repressive interactions. The left column shows Xenopus embryos that are stained for endogenous mRNA domains at neural plate stages to demonstrate the four ectodermal domains. *ker,* Epidermis; *six1*, preplacodal ectoderm (PPE); *foxD3*, neural crest; *sox2*, neural plate. A summary diagram of these domains is shown on top of the next column of embryos (color coding is the same as it was for Figures 27.1 and 27.3). In addition, the expression domains of two border genes *(zic2, dlx6)* are shown, and, on the bottom of that column, there is a summary of the relative expression domains for neural plate genes *(blue)*, neural plate border genes *(aqua)*, PPE genes *(green)*, and epidermis border genes *(orange)*. Experiments, which are summarized in the box to the right, demonstrate that the PPE gene, *six1,* may maintain preplacode fate in part through the mutual repression of genes expressed in the other domains and in the border zones of other domains. (Data from Brugmann et al., 2004, and from Brugmann and Moody, unpublished observations. (See color insert.)

1998; Fougerousse et al., 2002; Xu et al., 2003; Brodbeck and Englert, 2004; Himeda et al., 2004; Takasota et al., 2004), it is predicted that they will have distinct functions in PPE/placode development as well. Therefore, it will be important to perform both gain- and loss-of-function studies with *six2* and *six4,* both alone and in combination with *six1,* to better understand their functions in PPE and placode development.

B. *eya* Genes

There are four vertebrate *eya* genes that are homologues of Drosophila *eyes absent* (*eya*); the latter plays an essential role in fly eye development as a cofactor for *so* (Bonini et al., 1993). Vertebrate *eya* genes are expressed in multiple embryonic tissues, including the eyes, the somites, the kidneys, and the hypaxial muscle precursors; *eya1, eya2,* and *eya4* are also expressed in the PPE and the placodes (Abdelhak et al., 1997; Xu et al., 1997; Sahly et al., 1999; David et al., 2001). In Xenopus and zebrafish, *eya1* expression is remarkably similar to that of *six1* (Sahly et al., 1999; David et al., 2001), which suggests that it has an important role in PPE/placode development. The *eya* proteins do not bind to DNA, but they are characterized by a highly conserved protein/protein-binding domain called the *eya* domain (ED), which is located at the C-terminus of the protein. In Drosophila, the ED participates in protein/protein binding with the SD of the *so* protein (Pignoni et al., 1997), and, in vertebrates, the interaction between the *six1* SD and the *eya1* ED domains is essential for *eya1* nuclear translocation and for exerting the transcriptional function of the complex (Ohto et al., 1999; Ikeda et al., 2002). However, *eya1* can bind to several proteins in addition to *six1*. It can act as a cofactor for other *six* proteins (*six2, six4,* and *six5;* Heanue et al., 1999;

Ohto et al., 1999), and recent protein interaction studies in Drosophila identified several other potential *eya* binding partners (Giot et al., 2003; Database of Interacting Proteins Web site). Recent work has shown that *eya* functions as a phosphatase; this activity is necessary for Drosophila eye development (Rayapureddi et al., 2003; Tootle et al., 2003), and it is thought to regulate whether the *six1-Dach* complex (described later) acts as a transcriptional repressor or activator (Li et al., 2003). There is also evidence that *eya1* is a substrate for mitogen-activated protein kinase in the receptor tyrosine kinase signaling pathway (Hsiao et al., 2001). These potential multiple cellular functions will need to be kept in mind when evaluating the consequences of *eya* mutations in animal models and human congenital syndromes.

Experimental data for the role of *eya* proteins in PPE/placode development are most abundant for *eya1*. Several human cases have been identified that harbor *eya1* mutations, and these mutations cause some cases of BO and BOR syndromes (Abdelhak et al., 1997; Kumar et al., 1997; Rodriguez-Soriano, 2003; Spruijt et al., 2006), some cases of oto-facio-cervical syndrome (Rickard, 2001; Estefanía et al., 2006), and isolated defects in the anterior segment of the eye (Azuma et al., 2000). Often the defects lie in the ED, where they act to inhibit the interaction between *eya1* and *six* proteins (Buller et al., 2001; Ozaki et al., 2002). The *eya1* mutant mice show defects in the inner ear, some of the cranial ganglia, the thymus, the thyroid, the parathyroid, the kidney, and the skeletal muscles (Abdelhak et al., 1997; Johnson et al., 1999; Xu et al., 1997; 1999; 2002). The *dogeared (dog)* mutation in zebrafish (which is caused by a point mutation in the ED) and *eya1* knockdown by MO result in defects of the inner ear and of the lateral line, but the defects appear to affect the cell survival of the sensory cell precursors rather than to establish the PPE (Kozlowski et al., 2005). The analysis of the effects of two zebrafish *eya1* mutants *(aal* and *dog)* on the anterior pituitary, which is derived from the hypophyseal placode, showed that three of the four cell lineages are dependent on *eya1* expression but not *six1* expression (Nica et al., 2006). To date, mutations in *eya2* have not been reported in humans, and mutations in *eya4* are involved in nonsyndromic deafness, which is suggestive of developmental defects in the otic placode (Wayne et al., 2001; Zhang et al., 2004). The potential roles of these genes in PPE/placode development is ripe for further experimentation.

C. Are There Other *six/eya* Interacting Proteins Involved in Preplacodal Ectoderm Fate Specification?

It is well documented that *six* proteins bind, via their SDs, to several proteins that lack the ability to bind to DNA, and there is evidence that some of these proteins modulate *six* function as either coactivators or corepressors (Zhu et al., 2002; Tessmar et al., 2002; Giot et al., 2003). In fact, a gene network that includes *pax, six, eya,* and *fox* genes has been described to be essential in eye, lens, muscle, and kidney development (reviewed by Bhattacharyya and Bronner-Fraser, 2004; Brodbeck and Englert, 2004). In Drosophila, yeast two-hybrid experiments have identified 24 proteins in addition to *eya* that are likely to specifically bind to *so* (Giot et al., 2003; Kenyon et al., 2005). Because *so* belongs to the same *six* gene subfamily as vertebrate *six1/six2,* several of these proteins may have important roles in PPE fate specification. Recent work indicates that *six1/six2* can act as both transcriptional activators and repressors, depending on the presence of either *eya* or *groucho* cofactors

(Silver et al., 2003), and that these two proteins in combination with *six1* differentially influence PPE development (Brugmann et al., 2004).

As described above, *six1* expression in the Xenopus lateral neurogenic zone upregulates PPE marker genes and reduces the expression domains of genes that mark the adjacent epidermis and neural crest. To determine whether these effects of *six1* are executed via transcriptional activation or repression, activating *(six1VP16)* and repressing *(six1EnR) six1* constructs were expressed in the lateral neurogenic zone (Brugmann et al., 2004). These experiments demonstrated the following: (1) that *keratin* expression in the epidermis is repressed by *six1* both directly and indirectly, because wild-type *six1* and both activating and repressing constructs reduced its domain; (2) that PPE genes are transcriptionally activated by *six1,* because the effect of *six1VP16* mimicked wild-type *six1,* and the effect of *six1EnR* was the reverse; and (3) that *foxD3* expression in the neural crest is transcriptionally repressed by *six1,* because the effect of *six1EnR* mimicked wild-type *six1,* and the effect of *six1VP16* was the reverse. These interpretations are supported by experiments in which wild-type *six1* was coexpressed with either a known coactivator *(eya1)* or a known corepressor *(groucho)*. The coexpression of wild-type *six1* with *eya1* gave identical results to those obtained with the *six1VP16* construct for every marker gene examined, and the coexpression of wild-type *six1* with *groucho* mimicked the results obtained with the *six1EnR* construct. Because both *eya1* and *groucho* are endogenously expressed in the lateral neurogenic zone, these data indicate that *six1* functions in PPE development as both a transcriptional activator and a repressor, depending on the cofactor with which it interacts.

Because the Drosophila interactome data indicate that there are several other potential *six1* cofactors (Giot et al., 2003; Kenyon et al., 2005), there may be multiple modifiers of *six* transcriptional activity that are developmentally relevant to PPE/placode development. In addition, there are likely to be protein regulators that modify *six1* function by binding to or modifying the activity of *eya*. For example, *dac* has an important role in Drosophila eye development in cooperation with *eya* and *so* (Chen et al., 1997). *dac* can bind to both *eya* and DNA, but it does not have a direct interaction with *so*. Vertebrate Dach is expressed widely in embryonic tissues, including placodes (reviewed in Schlosser, 2006), and it can regulate the transcriptional effectiveness of *six/eya* complexes (Heanue et al., 1999; Ikeda et al., 2002; Li et al., 2003). However, a specific role for Dach or for other potential *six/eya* cofactors in PPE/placode development remains to be discovered. It is anticipated that the genome, proteome, and interactome databases of lower organisms (in particular *Caenorhabditis elegans* and Drosophila) will provide important clues regarding which additional factors are important in PPE/placode development. These types of investigations will allow for functional studies in vertebrate animal models, and they are likely to identify new causal genes in human congenital syndromes that affect the cranial sensory organs.

IV. MAINTAINING THE BOUNDARIES OF THE PREPLACODAL ECTODERM AND OTHER ECTODERMAL DOMAINS

As the neural plate is induced and the lateral neurogenic zone is established, several transcription factors become expressed along the neural plate border.

It has been proposed that some of these, which are called *neural plate border-specifying genes (dlx, msx, pax, zic)*, interpret the neural inductive and anterior–posterior signals in the locale of the lateral neurogenic zone and that they in turn activate neural crest fate-specifying genes (Meulemans and Bronner-Fraser, 2004). For example, *zic* genes, which are the vertebrate homologues of Drosophila *odd-paired,* become restricted to the lateral edges of the neural plate, and they are required for cranial neural crest formation (Nakata et al., 1997; 1998; Brewster et al., 1998; Kuo et al., 1998; Mizuseki et al., 1998). However, it is possible that these genes additionally function to maintain the boundaries between the various ectodermal domains by interacting with genes expressed in the adjacent domains. In chick, for example, *msx1* and *pax7,* which are required for neural crest formation, are first expressed in a broad stripe that becomes restricted to the neural folds as PPE genes are expressed in the lateral part of that stripe. This observation suggests that interactions between *msx1*/*pax7* and PPE genes lead to the segregation of fate domains (reviewed by Streit, 2004). Refinements in the domains of the genes that are first expressed in overlapping zones and then expressed in discrete stripes around the neural plate also have been described in Xenopus (reviewed by Schlosser and Ahrens, 2004; Schlosser, 2006). This pattern of broad, overlapping zones resolving to discrete domains via mutually repressive interactions is reminiscent of the establishment of segmental boundaries in Drosophila (see Chapter 9).

To date, only a few of the neural plate border–specifying genes have been investigated for their potential roles in affecting PPE formation. As discussed previously, *dlx3, dlx5,* and *dlx6* appear to be necessary for the initial formation of the lateral neurogenic zone and for the expression of both neural crest and PPE markers. The domains of these genes later resolve into stripes that border the PPE by late neural plate stages. The increased expression of either all three genes (Woda et al., 2003) or of *dlx5* or *dlx6* singly (Brugmann et al., 2004) in the lateral neurogenic zone of intact Xenopus embryos reduced the size of the PPE. Increased *zic2* expression also repressed the PPE while expanding the neural crest domains, and, interestingly, *six1* in turn repressed the expression domains of *dlx5, dlx6,* and *zic2* (Brugmann et al., 2004). These results suggest that at least some neural plate border–specifying genes *(dlx, zic)* and at least one placode fate-specifying gene *(six1)* mutually interact to maintain separate ectodermal domains. In support of this idea is that fact that other genes expressed in the various domains (e.g., *foxi1* in epidermis, *sox2* in neural plate, *foxD3* in neural crest) also have mutually repressive interactions with *six1* (Brugmann et al., 2004; Matsuo-Takasaki et al., 2005). Thus, after the fates of the four major ectodermal domains are specified by the expression of region-specific fate-specifying transcription factors, these factors may continue as maintenance factors to preserve the boundaries between these domains (see Figure 27.4).

However, it should be kept in mind that the types of experiments that have been performed to date do not sufficiently control the timing or spatial localization of overexpression and loss of function. Many of these genes likely have changing roles as the embryonic ectoderm becomes specified to different regional fates. For example, *foxi1* is initially expressed throughout the nonneural ectoderm, and it is required for the expression of later PPE marker genes (Matsuo-Takasaki et al., 2005). However, during neural plate stages when the PPE is fully established, the *foxi1* expression domain is mostly

lateral to those of *six* and *eya*, and overexpression at this time, which is controlled by a hormone-inducible construct, represses them. Thus, early *foxi1* expression may be required to specify the lateral neurogenic zone, and later expression may maintain the border between the PPE and the epidermis. Further manipulations involving the use of constructs that can be temporally and spatially controlled need to be performed to fully understand the molecular interactions that both establish and maintain the boundaries between the different ectodermal zones.

V. PLACODE IDENTITY AND ONSET OF DIFFERENTIATION

After the PPE is established as a separate domain in the embryonic ectoderm with distinct boundaries from the other ectodermal domains, the tissue undergoes several steps of differentiation. First, under the inductive influences of underlying tissues, the PPE subdivides into individual placodes with different fates (see Figures 27.1 and 27.2). Concomitantly, the placodes express different sets of transcription factors that likely reflect their acquisition of identity. The placodes then undergo the morphogenetic movements that will produce their wide range of sensory organ structures and cellular phenotypes. What is known about how these steps are accomplished is reviewed in detail elsewhere (Baker and Bronner-Fraser, 2001; Schlosser and Ahrens, 2004; Schlosser, 2005; 2006). Here, we focus instead on four sets of genes that are expressed in nearly every placode during the initial steps of differentiation in the following temporal order: (1) *six* and *eya*; (2) *sox*; (3) *pax*; and (4) proneural determination and differentiation (*basic-Helix-Loop-Helix [bHLH]*) genes (Figure 27.5). Do these genes constitute a regulatory network that controls the onset of placode differentiation?

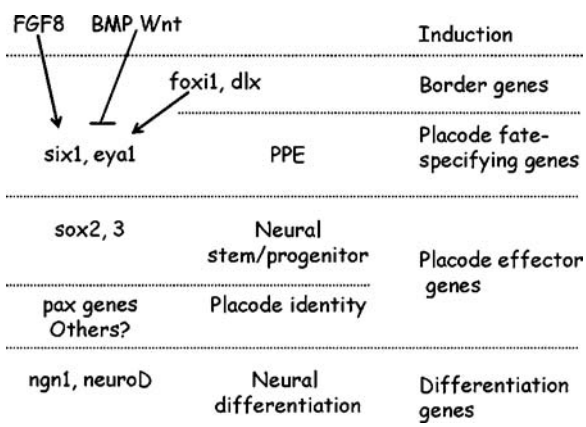

FIGURE 27.5 A model of the gene regulatory cascade that may regulate preplacodal ectoderm (PPE) formation and initial placode differentiation. First, a combination of signaling factors from the neural plate (fibroblast growth factor 8), the ventral epidermis (bone morphogenetic protein), the posterior end of the embryo (Wnt), and the epidermis border (regulated by *foxi1* and *dlx* genes) cause the formation of the PPE and the expression of the *six* and *eya* genes. Individual placodes form as *sox* genes, which may regulate the production of neural stem and progenitor cells in the neurogenic placodes, and *pax* genes, which may regulate placode identity, are expressed. As differentiation is initiated, neural progenitors express *neurogenin*-type genes; the subsequent expression of *neuroD*-type genes defines the precursors of different neural cell types.

During the initiation of placode separation, *six, six2, and six4* expression is maintained in all of the individual placodes except the lens (Pandur and Moody, 2000; Ghanbari et al., 2001; Schlosser and Ahrens, 2004); lens expresses *six3* instead (reviewed by Bhattacharyya and Bronner-Fraser, 2004). The *eya* genes are also expressed in all placodes, with some variation, depending on the animal and the placode (reviewed by Schlosser, 2006). Because *six* and *eya* genes continue to be expressed in all placodes, they are unlikely to be involved in the acquisition of the identity of the individual placodes. Instead, there is evidence that they are involved in the regulation of the initiation of differentiation. Expression patterns suggest that *six1* might maintain undifferentiated placodal cells in a "stem/progenitor" state (Brugmann and Moody, 2005). First, *six1* is downregulated as placodal cells show morphologic signs of differentiation, such as delaminating from the epithelium and coalescing into ganglia (Pandur and Moody, 2000). Second, *six1* expression is complementary to that of *bHLH* differentiation markers (*ngn1* and *neuroD;* Schlosser and Northcutt, 2000), which suggests that *six1* may need to be downregulated for placodal cells to differentiate. By contrast, *six2, six4,* and *eya1* continue to be expressed in differentiating placode-derived structures, including the cranial ganglia, which suggests that they may have later roles in placode differentiation, as has been suggested for mouse *six4* in other tissues (Niiya et al., 1998; Ohto et al., 1998).

Because *six1* is expressed earlier than *sox* and *pax* genes in the PPE and the placodes and because it is expressed earlier than and in a complementary pattern to *bHLH* genes during placode differentiation, its function after establishing the PPE fate may be to maintain subsets of placode cells in an undifferentiated state by repressing these other genes. If this is true, then *six1* gain of function should repress genes that are involved in initiating differentiation (see Figure 27.5), and it should promote continued cell proliferation. Preliminary data from our laboratory indicate that the overexpression of *six1* after the establishment of the PPE (using a hormone-inducible construct) reduces the expression of several of these later-expressed placode genes (Brugmann and Moody, unpublished observations). Studies in other systems indicate that *six* genes keep progenitor cells in a proliferative state before cell type specification. The loss of *six1* in mice appears to decrease proliferation, which results in apoptosis (Li et al., 2003; Ozaki et al., 2004). In humans, *six1* overexpression occurs in hyperproliferating cell populations (e.g., primary breast cancers and metastatic lesions; Ford et al., 1998). These authors showed that human *six1* overexpression allows DNA damage to go unchecked by causing an attenuation of the DNA damage–induced G_2 checkpoint. In a more recent study, the overexpression of *six1* was shown to influence cellular proliferation by directly activating the transcription of cyclin A1, a tissue-restricted cyclin that is expressed in the embryonic mammary gland but not in the differentiated adult mammary gland (Coletta et al., 2004). These studies suggest that *six1* may maintain cells in an immature state by influencing cell cycle regulation.

sox genes have been generally described as playing a role in the initial specification of the neural plate and neural stem cells (reviewed by Wegner, 1999; Moody and Je, 2002). Several experiments in several vertebrates indicate that *sox2* functions to maintain the neural stem cell state, and its premature inhibition causes neural cells to prematurely delaminate from the proliferative zone,

exit the cell cycle, and terminally differentiate (Mizuseki et al., 1998; Kishi et al., 2000; Graham et al., 2003). Although *sox2* and *sox3* are well-known markers of the vertebrate neural plate, they are additionally expressed in subdomains of the PPE after the onset of *six1* and *eya1* expression before the morphologic segregation of the individual placodes (Schlosser and Ahrens, 2004). It is possible that the *sox* genes are only expressed in the neural stem/progenitor cells of the neurogenic placodes and that they are thus responsible for the initiation of a neural differentiation pathway. However, *sox2* and *sox3* also play important roles in lens placode development, which has no neural derivatives (Kamachi et al., 1998), and they are not expressed in the profundal or trigeminal placodes, which give rise to sensory neurons (Schlosser and Ahrens, 2004). Therefore, they obviously have other functions in nonneural cells, and there must be other genes that initiate neurogenesis, at least in some placodes. One potential candidate is *sox11,* which is expressed in both the neural plate and PPE and which is upregulated by *six1* (Brugmann et al., 2004).

Members of the *pax* gene family have multiple roles in early developmental processes and organogenesis (Mansouri et al., 1999). In neural crest development, *pax3* and *pax7* have a role in specifying the neural plate border and, subsequently, the neural crest fate (Meulemans and Bronner-Fraser, 2004); whether they have a role in PPE specification has not yet been tested. However, after the PPE forms, several *pax* genes are expressed in restricted regions just before the placodes begin to separate (Baker and Bronner-Fraser, 2001; Schlosser and Ahrens, 2004). Individual placodes express different combinations of *pax* genes: *pax2, pax5,* and *pax8* are expressed in the otocyst; *pax6* is expressed in the olfactory, lens, and trigeminal placodes; and *pax3* is expressed in the ophthalmic/profundal placode. Transplantation experiments in the chick indicate that the onset of *pax* expression correlates with the acquisition of placode identity (Baker et al., 1999; Baker and Bronner-Fraser, 2000), thereby leading these authors to propose that the combination of *pax* genes expressed by an individual placode (the "*pax* code") may provide identity to that placode. However, many other transcription factors are also differentially expressed during the period when placodes separate, which suggests that they may also influence placode identity (Baker and Bronner-Fraser, 2001; Schlosser and Ahrens, 2004; Schlosser, 2006). For example, some early genes are expressed rather ubiquitously through the PPE zone (e.g., *six1, six4, eya1, dlx3*) but become repressed in the lens placode; this loss of expression may contribute to the lens fate. Other early genes (e.g., *Xiro1, foxi1, tbx2*) become restricted to only the most posterior placodes during placode separation, and thus they may influence posterior placode identities. There are many examples from loss-of-function studies that identify the genes that are required for the formation of individual placodes (Baker and Bronner-Fraser, 2001; Schlosser and Ahrens, 2004; Schlosser, 2006). However, because many of these genes are expressed both broadly during early stages and in specific placodes during later stages, it is difficult to determine whether the genes are involved in initial placode identity or in later differentiation processes. This will be an important issue to address using temporally and spatially controlled constructs.

Finally, *bHLH* transcription factors, which were first identified in Drosophila for playing essential roles in neurogenesis (reviewed in Jan and Jan, 1993; Guillemot, 1999), promote the generation of neural progenitors, cause neural progenitors to exit the cell cycle, and promote neuronal differentiation

(Lee et al., 1995; Bertrand et al., 2002; Ohnuma and Harris, 2003). They can be grouped into two classes: those that are expressed early in the neural fate cascade (the determination factors such as *neurogenin* [*ngn*]) and those that are expressed later in the cascade (the differentiation factors, such as *neuroD*). The expression of *ngn1* and *neuroD* in placodes and placodal derivatives has been extensively studied in Xenopus (Schlosser and Northcutt, 2000; Schlosser and Ahrens, 2004). In several placodes, *ngn1* expression is detected first in the inner ectodermal layer as soon as the individual placodes form, and later in the prospective ganglion cells that are delaminating and migrating away from the placode; *ngn1* expression is lost as the coalescing ganglion cells differentiate into neurons and glia. The expression of *neuroD* occurs later than that of *ngn1*; it is first seen in scattered cells within the inner ectodermal layer, and it remains expressed in most or all of the placode-derived ganglion cells. This sequence supports the general notion that *ngn1* acts early in the differentiation pathway and that it is followed by *neuroD*. Several studies suggest that neurogenic *bHLH* factors play important roles as regulators of neural differentiation in placodes after the acquisition of placode identity (see Figure 27.5; reviewed in Schlosser, 2006). The identity of similar factors for the nonneural derivatives of the placodes has not yet been studied in detail.

This simplistic scheme of gene expression sketches out a potential gene regulatory network for the initial steps of PPE/placode development (see Figure 27.5), but many details are still missing. By contrast, a gene network for neural crest specification has been proposed (Meulemans and Bronner-Fraser, 2004). First, BMP, Wnt, and FGF signals are necessary for the initial induction of neural crest fate. Next, these growth factor signals activate neural plate border–specifying genes, which in turn activate neural crest fate-specifying genes. These then regulate a large number of neural crest effector genes, which regulate differentiation pathways. Is there a similar gene network that regulates placode development and the onset of differentiation? First, PPE induction involves many of the same signaling molecules, but, whereas neural crest requires posteriorizing signals, these repress PPE. Second, there is evidence that some of the neural plate border-specifying genes that promote neural crest negatively regulate the PPE (see Figure 27.4). Third, the one PPE fate-specifying gene that has been studied to date *(six1)* seems to regulate at least some placode identity genes (*sox, pax;* perhaps these can be considered to be "placode effector" genes) and "neural differentiation" genes (*ngn1, neuroD;* see Figure 27.5; Brugmann and Moody, unpublished observations). Clearly, however, a great deal of work remains to be done to identify the full list of genes that are involved at each step of this putative network and to determine precisely how they interact to regulate the various aspects of PPE and placode development.

VI. FUTURE DIRECTIONS

Determining the molecular mechanisms of placode gene function is important for both understanding normal development and interpreting human congenital syndromes. First, the differential roles of all of the *six* proteins in PPE/placode development need to be determined. All three *(six1, six2, six4)* are expressed during placode development, and one affected locus (BOS3) in

BO and BOR patients contains *six1, six4,* and *six6* (Ruf et al., 2004). Second, a comprehensive understanding of which genes are able to interact with *six* genes as cofactors is needed. The penetrance of BO and BOR syndromes is variable, and studies in *six1* heterozygous mice suggest that there are additional modifier genes that influence *six1* activity or function, thereby modulating the mutant phenotype (Xu et al., 2003; Ruf et al., 2004). The recent interactome data from *C. elegans* and *Drosophila* indicate that there are several proteins yet to be experimentally tested that could potentially influence *six* and *eya* functions. Third, identifying and understanding the function of all of the genes involved in PPE specification and placode differentiation pathways will have a major impact on craniofacial tissue repair efforts. Elucidating the basic molecular mechanisms by which PPE cells are induced and transformed from the embryonic ectoderm into numerous differentiated cell types and how the process differs from that described for the closely related neural crest will be critical for designing techniques for sensory organ replacement from various stem and progenitor cell sources. Although we are at the very beginning of identifying the mechanisms that regulate PPE specification and initial placode differentiation, future work to elucidate the gene regulatory pathways reviewed herein may make it possible to repair craniofacial defects that result from birth defects, trauma, and disease.

SUMMARY

- The cranial sensory placodes arise from a PPE that is lateral to the anterior neural plate, and they give rise to a large number of specialized sensory organs.
- Three steps are necessary to induce and appropriately position the PPE: (1) interactions between the neural plate and the epidermis, perhaps involving FGF8; (2) the appropriate level of neural inductive (anti-BMP) signaling; and (3) the repression of posteriorizing signals (Wnt, FGF).
- The *six* and *eya* genes play important roles in specifying the PPE fate. In particular, *six1* positively regulates the expression of other PPE markers and negatively regulates the adjacent neural crest and epidermis. Importantly, *six1* does so both as a transcriptional activator and a transcriptional repressor, depending on the available cofactors, including *eya1* and *groucho*.
- Interactions between neural plate and epidermis border-specifying genes (e.g., *zic, dlx, foxi1*) and PPE genes refine the borders of the various ectodermal domains (i.e., epidermis, PPE, neural crest, neural plate).
- The *six* and *eya* genes may function upstream of putative neural stem genes *(sox)*, placode identity genes *(pax)*, and neural differentiation genes *(bHLH)* to regulate the onset of placode identity and differentiation.

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GLOSSARY

Adenohypophysis
The anterior pituitary gland, derived from the hypophyseal placode, that has cells that secrete a number of peptide hormones.

Branchio-otic syndrome
An autosomal-dominant syndrome in humans that presents with branchial cleft fistulas and hearing loss. Some cases are caused by mutations in the *eya1* gene, and some are caused by mutations in the *six1* gene.

Branchio-otic-renal syndrome
An autosomal-dominant syndrome in humans that presents with craniofacial defects, hearing loss, and renal or urinary tract defects. Some cases are caused by mutations in the *eya1* gene, and some are caused by mutations in the *six1* gene.

Lateral neurogenic zone
A region of the embryonic ectoderm that surrounds the anterior neural plate and that gives rise to the neural crest and to the preplacodal ectoderm.

Neurulation
The process by which the flat, disc-shaped neural plate ectoderm folds into an elongated tube, thereby becoming the precursor tissue of the central nervous system.

Organizer
The region of the vertebrate embryo that becomes the source of signaling molecules that dorsalize both the mesoderm and the ectoderm.

Oto-facio-cervical syndrome
Patients present with hearing loss; a long, narrow face; and various facial and cervical structural anomalies. Some cases are caused by mutations in the *eya1* gene.

Preplacodal ectoderm
The region of the embryonic ectoderm that surrounds the anterior neural plate and that is characterized by the expression of the *six* and *eya* genes, which will give rise to all of the cranial sensory placodes.

Stomodeum
An anterior region of the embryonic ectoderm that invaginates to contact the anteriormost end of the endoderm (i.e., the pharynx). This contact will eventually perforate and form the mouth.

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RECOMMENDED RESOURCES

Database of Interacting Proteins (DIP): http: dip.doe-mbi.ucla.edu/dip/Main.cgi.

Online Mendelian Inheritance in Man (OMIM): www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM.

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28 MOLECULAR GENETICS OF TOOTH DEVELOPMENT

IRMA THESLEFF

Institute of Biotechnology, University of Helsinki, Helsinki, Finland

INTRODUCTION

Teeth develop as appendages of the ectoderm, and their early development shares marked morphologic and molecular similarities with other ectodermal organs, such as hair, feathers, and many glands. Teeth are only found in vertebrates, but they are obviously not present in all vertebrates (e.g., birds). The ways in which dentitions are organized in different animals vary greatly. Most fish, amphibians, and reptiles have a homodont dentition (all teeth have a similar shape), and their teeth are replaced throughout life. Mammalian teeth, on the other hand, are sequentially organized into four groups from front to back: incisors, canines, premolars, and molars; they are mostly replaced once during the lifetime (primary or milk teeth are replaced by secondary teeth). The different tooth groups show characteristic differences in morphology (heterodonty). However, there are extensive modifications in the dental formulae within mammals, and, because the dentition is characteristic for each species, the variations in the patterning, numbers, and shapes of teeth have formed the basis for the analysis of fossil records and the understanding of mammalian evolution.

Most of the knowledge about the developmental anatomy of teeth and almost all of the knowledge of their developmental genetics has come from studies of mice. The central features of the morphogenesis of individual teeth are basically similar in all vertebrates, and, therefore, the information derived from mice is readily applicable to other animals and to humans. However, mouse dentition differs from that seen in most other animals in some aspects. First, they have only one incisor in each half of the jaw (humans have two incisors, and many other mammals have three), and the mouse incisors grow continuously. Second, although mice have three molars like humans and most other mammals, they completely lack cuspids and premolars. Third, most mammals have two dentitions, but mice have only one dentition, which is not replaced.

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There is a long tradition of research into the mechanisms of tooth development, and classic tissue recombination studies have shown that interactions between the epithelium and the underlying neural-crest–derived mesenchyme are instrumental regulators of tooth morphogenesis. On the basis of such studies and of some more recent work, it is now known that the epithelial–mesenchymal interactions are sequential and reciprocal and that they regulate both morphogenesis and the differentiation of the cells forming the dental hard tissues (Kollar and Baird, 1970; Lumsden, 1988; Thesleff and Nieminen, 2005). We are beginning to understand in greater detail the molecular and genetic bases of tooth morphogenesis as a result of the advances in gene technology and the availability of informative mouse models.

I. DEVELOPMENTAL ANATOMY

The initiation of individual teeth is preceded by the formation of an ectodermal ridge called the *dental lamina* (or *primary epithelial band*). In all vertebrates, the dental lamina forms as a stripe in the mandibular, maxillary, and frontonasal prominences of the embryo, and it marks the future dental arches. In the mouse embryonic jaws, the dental lamina thickens in the incisor and molar regions and forms placodes, which are multilayered epithelial condensations that resemble both morphologically and functionally the placodes of other ectodermal organs. The placodes then proliferate and form buds that intrude into the condensed dental mesenchyme (Figure 28.1). The transition of the bud to the cap stage starts when the epithelial bud invaginates at its

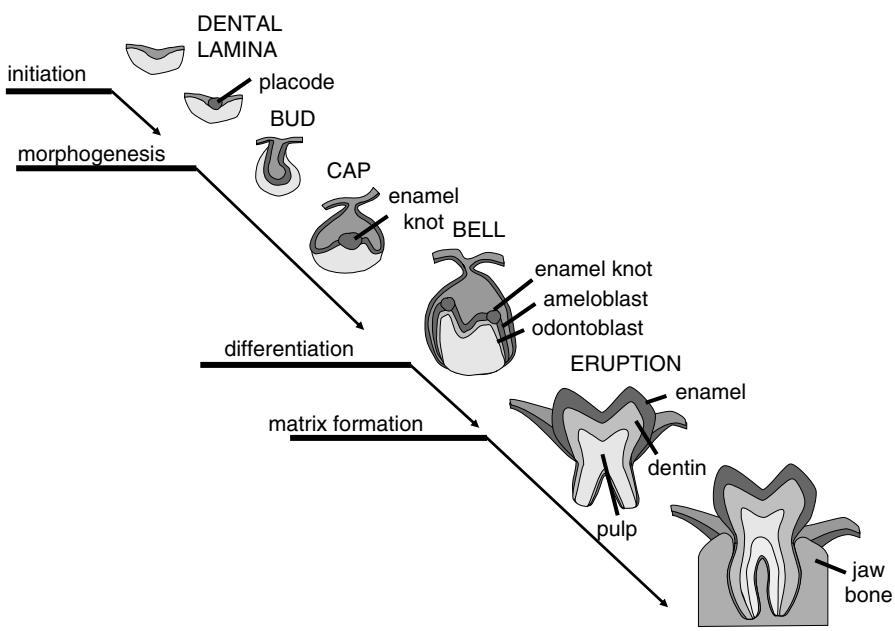

FIGURE 28.1 Development of a molar tooth. Interactions between the epithelium *(violet)* and the underlying mesenchyme *(yellow)* regulate development. The shape of the tooth crown is determined by the folding and growth of the epithelium. The placodes and enamel knots are signaling centers and important regulators of morphogenesis. (See color insert.)

tip. The enamel knot, which is a signaling center, forms at this location as an aggregation of epithelial cells, and it regulates the folding and growth of the epithelium. The epithelium flanking the enamel knot grows down, forming the cervical loops. The mesenchymal cells, which become surrounded by the epithelial cervical loops, form the dental papilla. These events determine the extent of the tooth crown. The epithelium differentiates into distinct cell layers and forms the enamel organ. The peripheral part of the condensed dental mesenchyme generates the dental follicle that surrounds the enamel organ epithelium and gives rise to periodontal tissues (Nanci, 2003).

During the following bell stage, the tooth germ grows rapidly, and the shape of the tooth crown becomes evident. The locations of cusps are determined by the secondary enamel knots. These form (similarly to the primary enamel knot) as epithelial thickenings, and they specify the points of epithelial folding. During the bell stage, the mesenchymal cells of the dental papilla directly underlining the dental epithelium differentiate into odontoblasts laying down the organic matrix of dentin, and the juxtaposed epithelial cells differentiate into ameloblasts depositing the enamel matrix (see Figure 28.1). Cell differentiation and matrix deposition always start at the tips of the future cusps (i.e., at the sites of enamel knots). During the entire morphogenesis of the tooth crown, a gradient of differentiation is seen, during which the stage of differentiation decreases in the cusptip-to-cervical direction.

The root forms after the completion of crown development in mouse molars as well as in all human teeth. Root morphogenesis is guided by the growth of the cervical part of the dental epithelium, and this epithelium does not differentiate into ameloblasts. Instead, the epithelium disintegrates, and this allows for contact between the dental follicle cells and the root surface and for their differentiation into cementoblasts secreting a thin layer of bone-like cementum. The dental follicle cells also form the periodontal ligament that links the tooth to alveolar bone. The tooth subsequently erupts into the oral cavity (see Figure 28.1).

Dentin resembles bone in its biochemical composition, although its histologic appearance is different. Unlike the bone-forming osteoblasts, the odontoblasts do not get incorporated into the dentin matrix. Instead, each odontoblast leaves behind a cytoplasmic process, which becomes embedded in dentin and thereby contributes to the formation of a dentin tubule. Odontoblast cell bodies remain as a confluent layer between the dentin and the cells of the dental pulp. The enamel matrix is composed of unique enamel proteins, including amelogenin, enamelin, and ameloblastin, which direct the formation and mineralization of enamel into the hardest tissue in the body. After the end of the secretory phase, the ameloblasts regulate the maturation of enamel, and they degenerate with the other layers of enamel epithelium during tooth eruption (Nanci, 2003).

II. GENE EXPRESSION PATTERN DATABASE

Dynamic expression patterns during tooth development have been reported for nearly 300 different genes, and this information has been collected to a graphic database (http://bite-it.helsinki.fi). Most of this information is derived from *in situ* hybridization analyses of embryonic mouse teeth, and, curiously, in the majority of cases, the tooth examined has been the lower (mandibular) first molar. Interestingly, most of the genes in the database encode molecules

that are associated with various signaling pathways. Although this certainly reflects the interests of the researchers in intercellular signaling, it conceivably is an indication that inductive cell interactions constitute the single most important mechanism regulating embryonic development (Gurdon, 1987; see Chapter 1).

The expression of most signal pathway genes is typically reiterated during advancing tooth morphogenesis (Jernvall and Thesleff, 2000). Most signal molecules belong to the four widely used conserved families: transforming growth factor (TGF)-β, fibroblast growth factor (FGF), Wnt, and hedgehog. With the exception of Shh, which is expressed only in epithelium, the signal molecules are expressed in both epithelium and mesenchyme, and the receptors are often seen in the adjacent tissue, which indicates that they may have roles in the mediation of tissue interactions. The gene expression patterns have suggested functions for all signal families in the regulation of the initiation, budding, and subsequent complex epithelial morphogenesis as well as in the differentiation of the dental cells, and many of the suggested roles have been confirmed in functional studies (Wang and Thesleff, 2006, review; also described later in this chapter).

Although the gene expression patterns are not direct indicators of functional significance, the information in the gene expression database has been—and continues to be—very useful in many ways. For example, the coexpression of several signal molecules in the enamel knots actually led to the unraveling of the function of the enamel knot as a signaling center (Jernvall and Thesleff, 2000). The database is used widely as a tool for examining the coexpression of genes and for the discovery of possible interactions between genes and molecules. Furthermore, as the mouse genome and the genomes of many other vertebrates have been sequenced, the gene expression database will have particular value as a tool for bioinformatics studies. Different sets of coexpressed genes can be selected and searched for common gene regulatory elements for the exploration of the principles of tissue-specific gene regulation.

III. THE DISRUPTION OF SIGNALLING PATHWAYS ARRESTS MOUSE TOOTH DEVELOPMENT

Tooth morphogenesis is arrested at an early stage in a large number of knockout mice, and, without exception, the reason for this is the blocking of one or more signal pathways. The first reported such mice were the *Msx1* null mutants, and the arrest in tooth development occurred at the transition from the bud to the cap stage (Figure 28.2; Satokata and Maas, 1994). *Msx1* is a transcription factor that functions both downstream and upstream of bone morphogenetic protein (BMP)-4, and its mutant phenotype was later rescued by BMP-4 (Bei et al., 2000). In *Pax9* null mutants, teeth are also arrested at the bud stage, and it was shown that *Pax9* is regulated by FGF and that it is also required for BMP-4 expression in the mesenchyme (Peters et al., 1998). The bud-to-cap stage transition is also blocked in *Lef1* and *Runx2* null mutant mice. *Lef1* is a transcription factor that mediates Wnt signaling, and its function is required in the epithelium, whereas *Runx2* functions in the mesenchyme. Both *Lef1* and *Runx2* mediate FGF signaling between the dental epithelium and the mesenchyme (Kratochwil et al., 2002; Åberg et al., 2004).

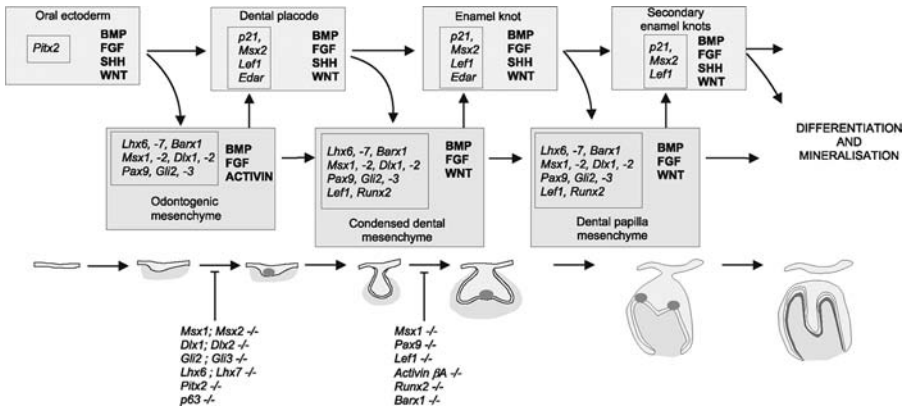

FIGURE 28.2 **Signaling networks regulating tooth morphogenesis.** The signals (marked with uppercase letters in the boxes: *BMP*, bone morphogenetic protein; *FGF*, fibroblast growth factor; *SHH*, sonic hedgehog; *WNT*) and their target genes (marked in italics in the boxes) expressed in epithelial *(white)* and mesenchymal *(grey)* tissues reiteratively mediate communication between or within the epithelium and the mesenchyme *(arrows)*. In the epithelium, the signals are mostly expressed in the signaling centers (the dental placode and the primary and secondary enamel knots, marked in black). Knockouts of several genes in mice (listed under the drawing) result in the arrest of tooth morphogenesis at the initiation or bud stage.

In many null mutant mice, tooth development is already arrested during initiation and before the formation of the dental placodes (see Figure 28.2). Such arrest in *Gli2/Gli3* double-mutant mice indicated a requirement for Shh signaling (Hardcastle et al., 1998), and a similar phenotype in *Msx1/Msx2* double mutants suggested a role of BMP signaling in tooth initiation (Satokata et al., 2000). The role of FGF signaling in early tooth development was indicated by conditional deletion of *Fgf8* in the oral epithelium, which resulted in tooth arrest during the initiation stage (Trumpp et al., 1999). The necessary role of Wnt signaling at this stage was demonstrated by the overexpression of the Wnt inhibitor Dkk1 in the ectoderm using the keratin 14 (K14) promoter (Andl et al., 2002). Taken together, the phenotypes indicate that all four conserved signal pathways are necessary already during the very early stages of tooth development (see Figure 28.2).

The development of all teeth is blocked in the previously described mutants, which indicates that the respective genes are required for the formation of all individual teeth. In the mouse, this means the single incisor and the three molars in each half of the two jaws. However, there are also examples of null mutant mice in which only some teeth fail to form, which suggests that there are differences in genetic regulation among different jaws and tooth types. When the function of the TGF-β signal activin is knocked out, all teeth except the maxillary molars are arrested before the bud stage, despite the fact that activin is expressed in all teeth (Ferguson et al., 1998). The *Dlx1/Dlx2* double mutants display the opposite phenotype, and they lack only the maxillary molars (Thomas et al., 1997). In the case of the *Dlx* null mutants, the likely reason is the redundancy of different *Dlx* genes, because *Dlx6* and *Dlx7* are additionally expressed in the maxilla, where they apparently rescue molar development. The transcription factors *Barx1, Lhx6,* and *Lhx7* are required for the development of molars but not for incisors, and the expression of these genes is restricted to molars (Grigoriou et al., 1998).

IV. THE GENETIC BASIS OF HUMAN TOOTH AGENESIS

The genes that were shown to be necessary for tooth development in knockout mouse studies have provided candidate genes in the search for gene mutations that cause human tooth agenesis. *Msx1* was the first gene that was shown to be required for mouse tooth development, and it was also the first gene identified behind human oligodontia (Vastardis et al., 1996). *Oligodontia* refers to severe tooth agenesis that affects more than six teeth, besides third molars (Thesleff and Pirinen, 2006). In addition to missing teeth, the *Msx1* null mice also have cleft palate, which is also associated with oligodontia in some human patients. *Pax9* is another gene that is required for mouse tooth formation, and it was linked with human tooth agenesis by the candidate gene approach (Stockton et al., 2000). Interestingly, although the *Pax9* null mutant mice have severe defects in a number of other organs as well, no additional defects have been reported in patients with human oligodontia caused by *Pax9* mutations.

The mutations that have so far been identified in the *Msx1* and *Pax9* genes are loss-of-function mutations, and these result in haploinsufficiency. The function of the respective genes is reduced, and hypodontia never affects all teeth in the affected individuals. The missing teeth typically represent the last teeth forming in the different tooth families; this feature characterizes all types of human tooth agenesis. However, there is a remarkable variation in the number of missing teeth between the patients with the same genotypes. The dental phenotypes of *Msx1* and *Pax9* mutations differ from each other in some aspects; the *Pax9* mutations particularly affect the molars. The human *Pax9* oligodontia phenotype was reproduced recently in mice by gradually reducing the gene dosage in an allelic series of *Pax9* mutant mice (Kist et al., 2005). It was shown that *Pax9* is required during multiple stages of tooth development, and that the minimal *Pax9* gene dosage required for the formation of individual teeth increased from the anterior to the posterior molars. These mice provide a useful mouse model for human oligodontia.

A third gene that has been linked with human oligodontia without associated congenital malformations is *Axin2*. Interestingly, instead of congenital defects, the patients were predisposed to colorectal cancer (Lammi et al., 2004). *Axin2* functions in the Wnt signal pathway as a feedback inhibitor, and it shares some functions with *APC,* which is the most common gene associated with colorectal cancer. The phenotype of oligodontia in these patients is different from those caused by *Pax9* and *Msx1* mutations, because *Axin2* mutations affect the secondary teeth almost exclusively. The normal development of deciduous teeth in the patients suggests that *Axin2* may be required for tooth renewal (described later).

V. DENTAL PLACODES AND THE PATHOGENESIS OF ECTODERMAL DYSPLASIA SYNDROMES

As described previously, tooth development is arrested in many knockout mice before the formation of the dental placodes. Because similar epithelial placodes initiate the development of all organs that form as appendages of the ectoderm (e.g., hair and nails; mammary, salivary, sweat, and sebaceous glands), it is not surprising that tooth defects are often associated with

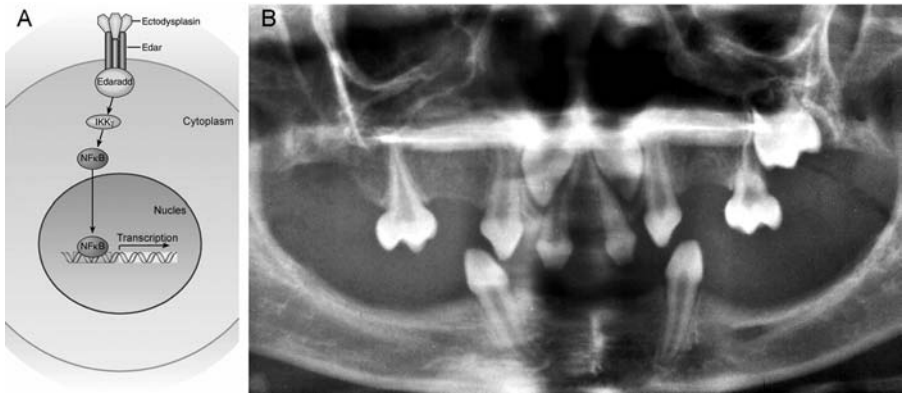

FIGURE 28.3 **Ectodysplasin regulates the development of all ectodermal organs. A,** The ectodysplasin–Edar signal pathway. Ectodysplasin is a tumor necrosis factor. The disruption of any one of the indicated genes in this pathway results in hypohidrotic ectodermal dysplasia. **B,** Severe tooth agenesis resulting from a loss-of-function mutation of the ectodysplasin gene.

congenital defects in other ectodermal organs (Pispa and Thesleff, 2003; Thesleff and Pirinen, 2005; Mikkola, 2007). It has become evident that the same genes regulate the formation and function of placodes in different ectodermal organs. Typically, signals in all of the four families are required for placode development. Studies mainly on hair and feathers have identified FGFs and Wnts as activators of placode formation and BMPs as inhibitors (Millar, 2002). The available evidence indicates that most of these functions are similar in dental placodes.

Ectodermal dysplasia syndromes are defined as conditions in which two or more types of ectodermal organs are affected, and dental defects in these syndromes typically include multiple missing teeth (oligodontia) and small and misshapen teeth (Figure 28.3). Many genes have been identified in which mutations cause ectodermal dysplasias. Given the similarities in the early development of various ectodermal organs, it is not surprising that these genes encode molecules that regulate placode formation and function. The analysis of the functions of the human ectodermal dysplasia genes in mouse models has elucidated the molecular mechanisms underlying ectodermal placode formation and increased the understanding of the pathogenesis of the human conditions.

Mutations in the transcription factor p63 cause EEC syndrome, which is characterized by ectodermal dysplasia, ectrodactyly, and cleft lip and palate (Celli et al., 1999). A typical patient has a severe dental phenotype with multiple missing and misshapen teeth. The *p63* knockout mice lack all ectodermal organs and die at birth (Mills et al., 1999). Detailed analysis of the tooth and hair phenotypes in these mice showed that development is arrested before placode development. The dental lamina forms normally as a normal multilayered epithelium, but the dental placodes fail to form (Laurikkala et al., 2006). Similarly, hair placodes are completely absent. It was shown that specifically the ΔN isoform of *p63* is required for the mediation of several signal pathways regulating placode formation. ΔNp63 function was necessary for FGF, BMP, and Notch1 signaling, and *FGFr2b, Bmp7,* and *Notch1* were identified as targets of *p63* (Laurikkala et al., 2006).

The positional cloning of genes behind hypohidrotic ectodermal dysplasia (HED) led to the discovery of the ectodysplasin (Eda) signal pathway, a novel tumor necrosis factor (TNF) pathway regulating ectodermal organ development (see Figure 28.3, A; Mikkola and Thesleff, 2003). The characteristic features of HED are oligodontia (see Figure 28.3), thin and sparse hair, and a severe lack of sweat glands; additional ectodermal defects in the nails and the salivary glands are also common. Mutations in the gene encoding the TNF ligand Eda cause the X-chromosomal form of HED, whereas mutations in the genes encoding the Eda receptor Edar and the signal mediator Edaradd are responsible for two autosomal forms of HED with a similar phenotype (Kere et al., 1996; Headon and Overbeek, 1999). Like other TNF signals, Eda signaling is mediated in the nucleus by the NFκB transcription factor (see Figure 28.3, A). HED-ID, a syndrome with all of the features of HED and associated immunodeficiency, is caused by impaired NFκB function as a result of mutations in IKKγ (NEMO), which is an intracellular key component in TNF signal pathways (Uzel, 2005).

The role of the Eda pathway in the development of teeth and other ectodermal organs has been studied in detail in mice (Mikkola and Thesleff, 2003). The mouse model for X-chromosomal HED, the *Eda* knockout mouse *(Tabby)*, has a tooth phenotype that is characterized by missing third molars and sometimes also incisors and by small and misshapen crowns on the first molars. The *Eda* null mouse also lacks the first wave of hair follicles and has defects in many ectodermal glands. Transgenic mice overexpressing Eda in the ectoderm have been informative with regard to the role of the Eda pathway and the pathogenesis of the ectodermal defects (Mustonen et al., 2003). Eda signaling is required for the formation and growth of ectodermal placodes. The Edar receptor is expressed in the placodes of all ectodermal organs, and, when it is overactivated, the placodes grow larger than normal. This results in the stimulation of ectodermal organ development that is seen as longer hairs, increased sweat excretion, extra mammary glands, and supernumerary teeth (Mustonen et al., 2003). The teeth form in front of the molars and may represent premolars, which were lost early during rodent evolution.

The application of Eda recombinant protein on embryonic mouse skin in culture rescues the hair follicle phenotype of *Eda* null mice. In wild-type embryos, Eda stimulates placode growth, and, in high concentrations, it causes fusions of the enlarged placodes. Interestingly, Eda does not stimulate cell proliferation but rather causes a change in the fate of ectodermal cells from an epidermal to placodal fate (Mustonen et al., 2004). The molecular mechanisms behind the function of Eda signaling have started to emerge through the recent discovery of the first direct target genes of Edar. Interestingly, these include two BMP inhibitors, CCN2 (CTGF) and follistatin, as well as Shh (Pummila et al., 2007). Hence, Eda signaling regulates placode formation and function by modulating the actions of the earlier known inhibitors (BMPs) and stimulators (Shh) of placode development (Millar, 2002; Mikkola, 2007).

Intriguingly, the injection of Eda protein to pregnant *Eda* null mutant mice rescued the hair and tooth phenotypes of their offspring (Gaide and Schneider, 2003). Ectodermal dysplasia is the first genetic malformation that has been permanently corrected by recombinant protein treatment in mice, and these findings may obviously lead to novel possibilities to prevent human X-linked HED in the future.

VI. ENAMEL KNOTS, TOOTH SHAPES, AND THE FINE-TUNING OF SIGNAL PATHWAYS

In addition to an arrest before placode formation, tooth development is blocked in many knockout mice at the late bud stage, before enamel knot formation and the transition to the cap stage (see Figure 28.2). Like the placodes, the enamel knots are signaling centers, and, in addition to the bud-to-cap stage transition, the enamel knots have a key role in the regulation of the shape of the tooth crown by the induction of secondary enamel knots initiating cusp development (Jernvall et al., 2000; Jernvall and Thesleff, 2000). Interestingly, there are also mouse mutants in which tooth development is not completely inhibited; rather, the shapes and sizes of teeth are abnormal as a result of patterning defects, and, in these mutants, the gene defects seem to affect the formation and function of enamel knots. The formation of the enamel knots requires the integrated functions of several stimulatory and inhibitory signals, and the functions of the enamel knots as signaling centers are carried out by the localized expression of at least a dozen different signal molecules that regulate tooth patterning.

The direct link between enamel knot formation and cusp patterning was first demonstrated in a study comparing embryonic tooth crown development between two rodents: the mouse and the vole (Jernvall et al., 2000). The enamel knots are initiated before morphologic development; their patterning predicts the positions of future cusps, and it is responsible for the markedly different crown configurations in the two species. A mathematic model was subsequently proposed on the basis of the interplay of stimulatory and inhibitory signals; this model can reproduce a variety of species-specific patterns of secondary enamel knots and cusps (Salazar-Ciudad and Jernvall, 2002). Functional evidence supporting this particular model has been gained from the tooth phenotypes of some mouse mutants, and recent studies have illustrated how the fine-tuning of the signal pathways may modulate dental patterning.

The phenotype of the *Eda* null mutant mice (described previously) is characterized both by missing teeth and the abnormal morphology of the first molars. The restricted expression of the Edar receptor in the placodes and enamel knots indicates that the dental defects are the result of the impaired functions of the signaling centers, and also that Eda signaling may affect several stages of tooth morphogenesis. The cusps of the first molars in *Eda* null mice are fused or missing. That this phenotype is the result of defective enamel knot signaling was suggested by the small size of the primary enamel knots and the fusion of the secondary enamel knots (Pispa et al., 1999). By contrast, mice overexpressing ectodysplasin in the ectoderm have large molar placodes and enamel knots that explain the extra teeth in front of the first molars and the abnormal cusp patterns (Mustonen et al., 2003; 2004; Kangas et al., 2004). The cusp pattern of the Eda-overexpressing mice curiously resembles that of kangaroos (Figure 28.4). This, together with the reduced cusps in the *Eda* null mice, indicates that changing the dose of a signal molecule can cause dramatic changes in teeth and profoundly alter the species-specific cusp pattern.

The findings that Eda signaling modulates at least two other signal pathways, BMP and Shh, suggest that the effects of Eda overexpression on tooth patterning result from fine-tuning the effects of other major signaling pathways (Pummila et al., 2007). Abnormal cusp patterns have also been detected

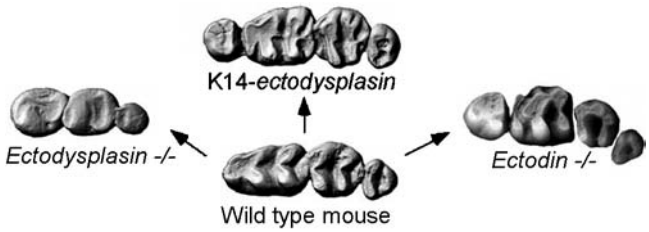

FIGURE 28.4 **Fine-tuning of cell–cell signaling alters dental patterning.** Molar teeth of transgenic mice show changes in tooth number, size, and cusp pattern. The ectodin null mutant has an extra tooth (premolar) and fused first and second molars, and the cusp pattern resembles that of a rhinoceros. The ectodysplasin null mutant has small teeth with reduced cusps (in addition, the third molar is often missing), and the transgenic mouse overexpressing ectodysplasin (K14–ectodysplasin) has an extra tooth and an altered cusp pattern that resembles that of a kangaroo. (Courtesy of Jukka Jernvall.)

in mice overexpressing the TGF-β inhibitor follistatin (Wang et al., 2004a) and in mice lacking the function of ectodin, a BMP inhibitor and Wnt modulator (Kassai et al., 2005). The first and second molars of the ectodin knockout mice are often fused and have dramatically altered cusp patterns, resembling the molars of the rhinoceros (see Figure 28.3). The phenotypes of the different mouse mutants suggest that cusp patterning is critically dependent on the balance of the different signal pathways. It has been proposed that evolutionary changes in cusp patterns in mammalian teeth may have involved the fine-tuning of signaling (Kangas et al., 2004; Kassai et al., 2005).

VII. THE GENETIC BASIS OF TOOTH REPLACEMENT

Although most mammals have two dentitions (the primary and secondary dentitions), the cellular and molecular mechanisms of tooth replacement have remained poorly understood (Huysseune and Thesleff, 2004). This is mainly because the mouse, which is practically the only model animal used thus far, does not have a secondary dentition. Recently, however, mouse mutants were produced that renew their teeth continuously. In these mice, Wnt signaling was activated in the embryonic ectoderm by the conditional expression of a stabilized form of β-catenin ($\beta cat^{ex3K14/+}$; Järvinen et al., 2006). The mice died at birth, and, at that time, the tooth buds were morphologically abnormal; however, the abnormalities were not indicative of new tooth formation. The remarkable phenotype became evident when the embryonic tooth buds were grown as transplants under the kidney capsules of nude mice. After 3 weeks, one tooth bud had generated more than 40 new teeth (Figure 28.5). Transplanted incisor tooth buds formed new incisors, molars generated molars, and the teeth represented different developmental stages, thus indicating continuous tooth production.

It was shown that the initiation of new teeth in the $\beta cat^{ex3K14/+}$ mice took place sequentially from new enamel knots that were induced in the dental epithelium of the earlier formed teeth. Histologic observations of tooth replacement in reptiles renewing their teeth continuously (as well as of human permanent teeth forming from deciduous teeth) have indicated that the successional teeth are initiated from the dental epithelium of their predecessors. Hence, the process that was activated in the $\beta cat^{ex3K14/+}$ mouse mutants

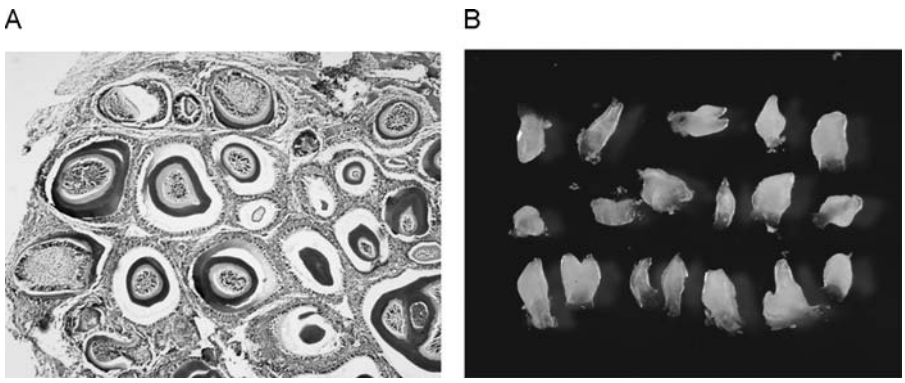

FIGURE 28.5 **Activation of Wnt signaling stimulates tooth renewal. A,** Histologic section through a tumor-like outgrowth that developed from one transplanted molar tooth bud of a mouse embryo expressing a stabilized form of β-catenin ($\beta cat^{ex3K14/+}$). Dozens of teeth have formed, and they represent different developmental stages. **B,** Mineralized teeth that were dissected from a similar outgrowth. The teeth have roots and mostly simple conical crowns. (See color insert.)

resembles physiologic tooth replacement. Because the continuous production of teeth in the mutants was caused by activated Wnt signaling in the epithelium, it was concluded that the capacity of tooth replacement depends on the activity of Wnt signaling (Järvinen et al., 2006).

Although the human teeth are normally replaced only once, there is a rare syndrome called *cleidocranial dysplasia* (CCD) that is characterized by multiple supernumerary teeth representing a partial third dentition (Jensen and Kreiborg, 1990). CCD is caused by loss-of-function mutations in one copy of the *Runx2* gene (Mundlos et al., 1997). The examination of the dental phenotype of the mouse model of CCD (i.e., *Runx2* heterozygote mice) showed that Shh-expressing extra buds form at the lingual aspect of the first molar (Wang et al., 2005). These resemble the successionally forming buds in the $\beta cat^{ex3K14/+}$ mutant mice, which were monitored by their Shh expression. Although *Runx2* is required for the mediation of FGF signaling during early tooth formation (Åberg et al., 2004), it also regulates Wnt signaling in dental mesenchyme (our unpublished results); hence, the role of *Runx2* in tooth renewal may well be associated with the Wnt pathway.

Additional support for the role of the Wnt pathway in human tooth replacement comes from the tooth phenotype caused by *Axin2* mutations (Lammi et al., 2004). The patients characteristically lack multiple permanent teeth, whereas their deciduous dentition is unaffected. Because *Axin2* participates in Wnt signaling in the cytoplasm and because it is also a direct Wnt target, it was suggested that tooth renewal may be regulated by Wnt signaling (Lammi et al., 2004). A role of Wnt signaling in tooth replacement would also be in line with similar functions of Wnts in hair cycling (Gat et al., 1998). Because the molecular mechanisms regulating the embryonic morphogenesis of different ectodermal organs are similar, it is conceivable that the mechanisms involved in adult regeneration are shared between teeth and other organs forming as appendages of the ectoderm (Huysseune and Thesleff, 2004). In conclusion, the current evidence indicates that the fine-tuning of Wnt signaling plays a key role in regulating tooth formation and renewal, and it was suggested that the reduced ability of tooth regeneration during

mammalian evolution has involved changes in Wnt signaling (Järvinen et al., 2006).

VIII. THE DEVELOPMENTAL GENETICS OF DENTIN AND ENAMEL FORMATION

Inherited defects in dentin and enamel structure are rare. Amelogenesis imperfecta refers to hereditary defects in enamel formation. Mutations have been identified in human patients with amelogenesis imperfecta in genes that encode enamel proteins, such as amelogenin and enamelin (Stephanopoulos et al., 2005). In addition, enamel defects occur as traits in several syndromes, mostly in association with skin diseases and metabolic diseases (Thesleff and Pirinen, 2005). Dentinogenesis imperfecta and dentin dysplasia are severe dentin defects that affect both the crown and the roots of the teeth, and mutations in the dentin matrix component dentin sialophosphoprotein have been identified as causes (Xiao et al., 2001). Mutations in type I collagen, which is the main component of bone and dentin matrix, cause osteogenesis imperfecta, which affects both bones and teeth. So far, mutations in regulatory genes involved in the formation of enamel and dentin have not been associated with amelogenesis imperfecta and dentinogenesis imperfecta. However, there is increasing evidence from mouse models that indicates that the interference with genes regulating cell differentiation causes defects of enamel and dentin.

Dentin and enamel are produced by columnar mesenchymal and epithelial cells: the odontoblasts and the ameloblasts, respectively (see Figure 28.1). Their differentiation is regulated by reciprocal epithelial–mesenchymal interactions, and the same signal molecules that are used for morphogenetic regulation have also been associated with dental cell differentiation. Signals in the TGF-β and FGF families (Unda et al., 2000) and recently also in the Wnt family (Yamashiro et al., 2007) have been implicated in odontoblast differentiation. BMPs were originally identified as inducers of bone, and their capacity to induce odontoblast differentiation and dentin matrix production has long been examined *in vivo* in attempts to promote dentin regeneration (Nakashima and Reddi, 2003). BMPs have also been associated with ameloblast differentiation, and direct evidence involving the function of BMP in this process was presented recently (described later). In addition, Shh signaling is required for the proper polarization of ameloblasts (Gritli-Linde et al., 2002), and it was shown that *Msx2* is required upstream of Shh in ameloblast differentiation (Bei et al., 2004).

The mouse incisor is traditionally used as a model to study the formation of dentin and enamel, because the incisors grow continuously, and the dental hard tissues form throughout the life of the animal. Recent evidence from the incisors of some transgenic mice indicates that BMP-4 is the major signal molecule that regulates ameloblast differentiation and enamel formation. Inhibition of BMP function by overexpression of either follistatin or noggin in the incisor epithelium prevents enamel formation (Wang et al., 2004b; Plikus et al., 2005). Follistatin also contributes to the characteristic asymmetry of enamel distribution in mouse incisors. Enamel forms only on the labial (anterior) surface in wild-type incisors, but, in follistatin knockouts, ectopic enamel formed on the lingual (posterior) surface of the incisors (Wang et al., 2004b). Hence, follistatin (which is expressed in the lingual epithelium) prevents the inductive function of BMP-4 on the lingual side of the incisor, and this results in a failure of ameloblast differentiation and a lack of enamel formation

lingually. This, together with the continuous growth, accounts for the maintenance of the sharp cutting edge of the mouse incisors.

The enamel phenotypes of the various transgenic mice resemble the enamel defects seen in some human syndromes. In addition, in most of these mouse mutants, the morphogenesis of teeth is disturbed, and this results in missing or extra teeth and aberrant tooth shapes. Examples are mice lacking Shh and *Msx2* expression and mice overexpressing ectodysplasin, Edar, or follistatin (Bei et al., 2004; Mustonen et al., 2003; Wang et al., 2004a; 2004b). Accordingly, in human syndromes, enamel defects are often associated with other dental anomalies, typically with the reduction of tooth number and size. These observations reflect the fact that the same genes regulate different aspects of tooth development (including initiation, morphogenesis, and the differentiation of the hard-tissue–forming cells) and that the genes are iteratively used during the advancing stages of tooth development. Furthermore, the congenital defects are mostly not limited to teeth either in these mouse mutants or in human syndromes. The additional malformations in other organ systems (mostly ectodermal organs) are obviously explained by the widespread expression patterns of the genes and the fact that the developmental regulatory genes have multiple functions in the embryo. These and other examples in this chapter underline the importance of understanding developmental genetics to unravel human syndromes.

SUMMARY

- The genes and mechanisms that regulate tooth development are remarkably similar to those that regulate other tissues and organs, particularly other organs that develop as ectodermal appendages. It is noteworthy that, so far, no regulatory gene unique to teeth has been identified. Therefore, severe abnormalities in tooth development are mostly associated with defects in other tissues and organs. In addition, there is one example of a syndrome in which the same mutation that causes tooth agenesis also promotes the malignant development of epithelium and creates a predisposition to colorectal cancer (*Axin2;* Lammi et al., 2004).
- It is mostly impossible to predict the causative gene from the phenotype of a dental defect. Different genes may have similar functions, and, in addition, many genes (particularly those participating in the mediation of cell–cell signaling) are used reiteratively during development. They regulate morphogenesis as well as cell differentiation, and their mutations may therefore affect the numbers, shapes, and hard-tissue structure of teeth.
- Most genes and molecules that regulate tooth development seem to be associated in some way with the four major signaling pathways: TGF-β, Wnt, Shh, and FGF. Such molecules include (in addition to the secreted signaling molecules themselves) receptors and other molecules involved in the mediation of the signals to the nucleus as well as the transcriptional targets of the signals and, very importantly, different types of modulators of signaling.
- Evidence underlining the importance of signal inhibition is increasing rapidly, and there are already many examples of the functions of the fine-tuning of signal pathways during tooth development. The morphogenesis and cell differentiation can be dramatically affected by increasing or decreasing the function of individual signal inhibitors.

- The different signal pathways are integrated at many levels, and they affect the functions of each other. As an example, recent work on the regulation of the stem cell niche in the continuously growing mouse incisors has pinpointed a complex cooperation of FGF, BMP, and activin signals. Their balance regulates epithelial stem cell proliferation and differentiation, and it determines the rate of incisor growth and the asymmetric distribution of enamel formation (Wang et al., 2004b; 2007).
- The modulation of the various signal pathways generates differences in tooth shapes and sizes, and it also determines the capacity to regenerate teeth as shown by the continuous tooth renewal induced by the activation of Wnt signaling in transgenic mice. It is conceivable that the fine-tuning of signal networks is the key mechanism that has regulated the development of the species-specific characteristics of the vertebrate dentitions during evolution.

GLOSSARY

Ectodermal dysplasias
Syndromes that affect two or more ectodermal organs; the most common syndromes involving dental defects.

Enamel knots
Key signaling centers that regulate tooth morphogenesis.

Hypodontia
Tooth agenesis (the congenital absence of one or more teeth).

Odontogenesis
Tooth development.

Oligodontia
Severe tooth agenesis that affects more than six teeth, besides third molars.

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RECOMMENDED RESOURCES

Tooth Gene Expression Database: http://bite-it.helsinki.fi

Online Mendelian Inheritance in Man: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM&cmd=Limits

Mouse Genome Informatics: http://www.informatics.jax.org/

29
THE INNER EAR

DONNA M. FEKETE and ULRIKE J. SIENKNECHT

Department of Biological Sciences, Purdue University, West Lafayette, IN

INTRODUCTION

The past 10 years have seen an explosive growth of information involving the molecular bases of inner ear induction and regionalization, cell-type specification, and the polarization of *hair cells* (HCs) in the vertebrate inner ear. Data from mechanoreceptive sensory organ development in model organisms such as the fly continually add to the list of candidate genes that require further study to enhance our understanding of vertebrate ear and HC development. The discovery that shared molecular programs may be used to generate widely divergent mechanoreceptive organs has rekindled interest in the evolutionary origins of hearing and balance. In some cases, knowledge of inner ear development in animals has led to the identification of human deafness genes, whereas, in other cases, information has flowed in the opposite direction, with deafness genes providing the first hint that a gene is important during embryogenesis to form the inner ear. Today, the parallel approaches of experimental embryology, molecular biology, and human genetics are synergizing to advance our understanding of human embryology and human congenital defects of the inner ear.

A. Anatomy of the Inner Ear

The vertebrate inner ear arises from the otic placode and then "morphs" from a simple vesicle into an elaborate three-dimensional array of chambers and ducts filled with potassium-rich endolymphatic fluid (Figure 29.1). All sensory organs of the inner ear contain mechanosensory HCs with their characteristic staircased bundles of stereocilia, accessory cells called *supporting cells,* and a variety of extracellular specializations lying above the organ that serve to couple vibration or fluid motion in the endolymph with motion of the HC bundles. When mechanically gated transduction channels open at the tips of the HC stereocilia, the potassium ions in endolymph carry the majority of the charge through the channels. The dorsal vestibular half of the ear is highly conserved: in nearly all vertebrates, three orthogonally arrayed *semicircular*

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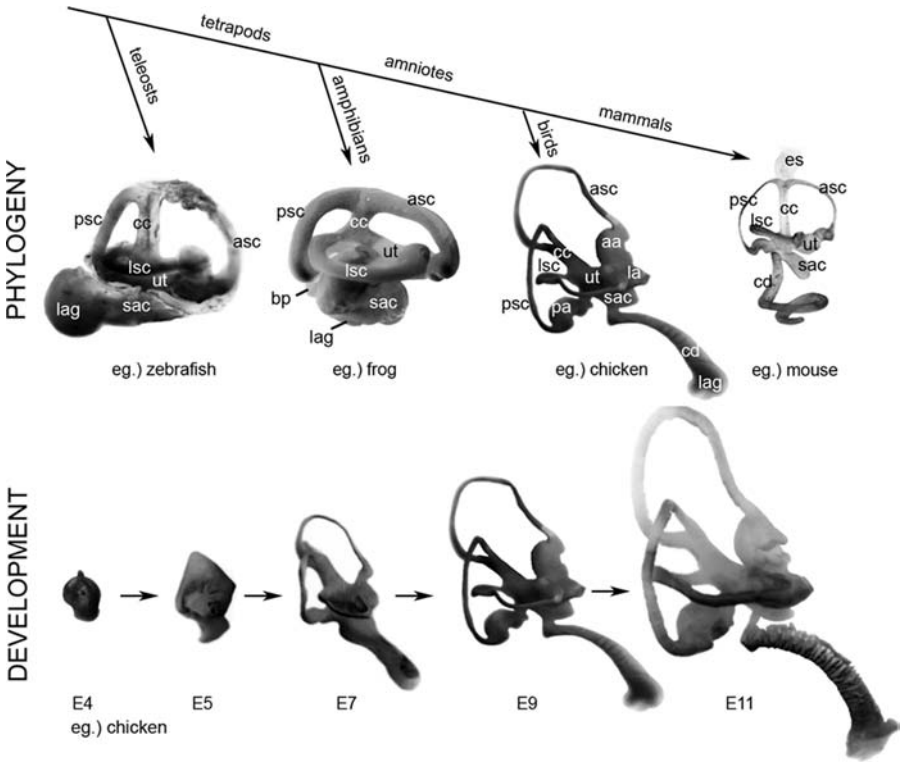

FIGURE 29.1 **Phylogeny *(top row)* and development *(bottom row)* of the vertebrate inner ear viewed by filling the endolymphatic cavities of the inner with opaque paint.** Specimens are shown from a lateral view. There are three semicircular canals and both utricular and saccular recesses in all four representatives of different chordates during gestation. Zebrafish (20 days), Xenopus (stage 49), chicken (embryonic day 9), and mouse (embryonic day 15) are shown. Adjacent to the saccule, the lagenar chamber is also fairly well conserved, and it may have elongated into the cochlear duct. The location of the hearing organs of birds and mammals supports the idea that it may have originated from the saccular macula, which is the hearing organ of most fishes and some amphibians. The lower row shows the morphogenesis of the inner ear of the chicken embryo as a representative vertebrate. Embryonic days *(E)* are indicated. *aa,* Anterior ampulla; *asc,* anterior semicircular canal; *bp,* basilar papilla; *cc,* common crus; *es,* endolymphatic sac; *lag,* lagena; *la,* lateral ampulla; *lsc,* lateral semicircular canal; *pa;* posterior ampulla; *psc,* posterior semicircular canal; *sac,* saccule; *ut,* utricle. (Modified from Morsli et al., 1998; Bever and Fekete, 2002; Bever et al., 2003; and Bissonnette and Fekete, 1996.)

canals are present, and each of them ends in an enlarged ampullary sac that houses a sensory *crista*. These organs sense angular acceleration. Two of the canals (the anterior and posterior canals) meet in the middle of the ear at a duct called the *common crus,* which then merges with the utricular recess. The central chambers of the ear—the utricle and the saccule—each house a macula. These sensory organs mediate the sense of linear acceleration (gravity). The ventralmost portion of the vertebrate inner ear may extend as a *cochlear duct*. When present, the cochlear duct houses the auditory receptor organ, which is called the *organ of Corti* in mammals and the homologous *basilar papilla* in other amniotes (birds, crocodiles, snakes, and lizards). In other species, hearing is mediated by additional papillae (in amphibians) and/or

one or more of the macular organs (e.g., in cartilaginous and bony fishes), as discussed later. In summary, as one looks across the vertebrate inner ears, semicircular canals, utricles, and saccules are the most conserved features (see Figure 29.1). Also conserved are a pair of nonsensory elements called the *endolymphatic duct* and *sac* (shown only for the mouse ear in Figure 29.1) that allow endolymph to percolate into the cerebrospinal fluid. The endolymph-filled compartments are surrounded by perilymph, which has an ionic composition that is more akin to normal extracellular fluid (high sodium, low potassium). The perilymphatic system is itself surrounded by a bony or cartilaginous *otic capsule* that protects the delicate inner ear and that completes the catalog of structural elements comprising a "typical" vertebrate inner ear.

B. Human Deafness Genes and Inner Ear Development

Animal models have provided important clues about the underlying causes of congenital deafness in humans. More than 62 human deafness genes, including mitochondrial genes, have now been identified (Van Camp and Smith, 2007). Of these, the structures or functions affected by the mutations (at least in animal models) run the gamut from early ear morphogenesis to stereociliary bundle development to tissue differentiation and the regulation of the ionic milieu of the endolymph. Many of the identified genetic mutations underlie syndromic forms of deafness, in which hearing loss is found in the context of defects in other organ systems. Syndromic deafness can be associated with genes that function during early stages of development, when inner ear tissue is being patterned and cell fates are being allocated. Examples include *EYA1* and *SIX1,* which are two genes that underlie the branchiootorenal syndrome, and there are six different genes that are responsible for Waardenburg syndrome. Other genetic mutations are associated with nonsyndromic deafness, and often these are linked to cellular functions that are rather unique to the inner ear. For example, at least a dozen human deafness genes code for proteins involved in stereocilia bundle development and/or HC function, including cytoskeletal components, submembranous scaffolds, unconventional myosin motors, cell adhesion proteins, ion channels, and extracellular matrices that mechanically couple stimuli to bundle deflection (Frolenkov et al., 2004; Kelley, 2006). Maintenance of the ionic environment of the endolymph is also a site of vulnerability to genetic mutations, some of which are specific to the inner ear (nonsyndromic), whereas others are required by other organs, such as the heart or kidney. Genes underlying ionic homeostasis include those encoding ion channels, transporters, and gap junctions, which provide an intercellular conduit for the extensive recycling of potassium ions needed to replenish endolymph (Wangemann, 2002). These examples illustrate how an understanding of both development and cell biology of the inner ear can reveal the root causes of congenital human deafness.

I. EVOLUTION OF MECHANORECEPTORS

The central element for the detection of sound, vibration, or relative movement is the *mechanoreceptor.* In the context of hearing and balance, we are most

interested in mechanoreceptors that possess apical surface specializations, which provide a structural basis for sensing near-field particle movement (e.g., HCs of the lateral line neuromasts) or far-field pressure waves in either air or water. Tracing mechanoreceptor lineages back in time to reveal the morphology of an archetype to the vertebrate HC is not trivial for two reasons: the diversification of receptor types and the strong functional dedication of mechanosensory cells. The functional constraints of mechanosensation may blur the discrimination between true homology (i.e., descendants of a common ancestral cell type) as compared with resemblance as a result of convergent evolution (Manley and Ladher, 2007). Although molecular genetics provides new insights regarding similarities across distantly related species, it is necessary to carefully establish criteria for molecular homology. With these caveats in mind, molecular genetics may help to resolve some of the longstanding controversies regarding the evolution of hearing organs, which it is beginning to do for other organ systems like hearts and eyes.

A. Primary Versus Secondary Mechanoreceptor Cells

Sensory cells with structural similarities to vertebrate HCs are found in quite different groups of organisms ranging from tunicates (sea squirts) to molluscs to basal eumetazoans, including the cnidarians, such as jellyfish (Coffin et al., 2004; Holland, 2005). The vertebrate HC is a specialized epithelial cell called a *secondary mechanoreceptor,* because it does not possess its own axon but rather its cell body synapses onto projection neurons that relay the information to the brain. Secondary mechanoreceptors are also found in chordate tunicates and in some cephalopods (octopus and its relatives*)*. This is in contrast with the primary mechanoreceptors, which are mainly found in cephalopod molluscs as well as in other major invertebrate groups (e.g., arthropods), including Drosophila. A *primary mechanoreceptor* possesses its own axon, and it is generally assumed to be evolutionarily older than the secondary type (Fritzsch et al., 2006). For many years, the presence of secondary mechanoreceptors in both chordates (i.e., tunicates, lancelets, and vertebrates) and derived cephalopod mollusks (e.g., octopus) was argued to be a case of convergent evolution (Coffin et al., 2004). However, evidence for a shared requirement for *Atonal*-like genes in the development of mechanoreceptor progenitors in both vertebrate and invertebrate model organisms now suggests a deep evolutionary homology in the origin of mechanoreceptors (described later).

B. Ciliated Versus Microvillar Mechanoreceptor Cells

In addition to subdividing mechanoreceptors as either neuronal (primary) or epithelial (secondary) cell types, they can also be grouped as ciliated or microvillar according to their apical specializations (Figure 29.2). In most invertebrates, the apical surface of the mechanoreceptor cell is adorned with at least one apically protruding microtubule-based cilium that is an intimate part of the mechanosensory transduction apparatus. Although vertebrate HCs also possess a true cilium called the *kinocilium,* their apical surface is dominated by rows of stiff, actin-rich membrane protrusions that are modified microvilli. These *stereocilia* are staircased into graded heights of bundles (or "hairs") possessing mechanosensitive ion channels that are associated with the "tip link" of each stereocilium. Thus far, only the chordates are known to have

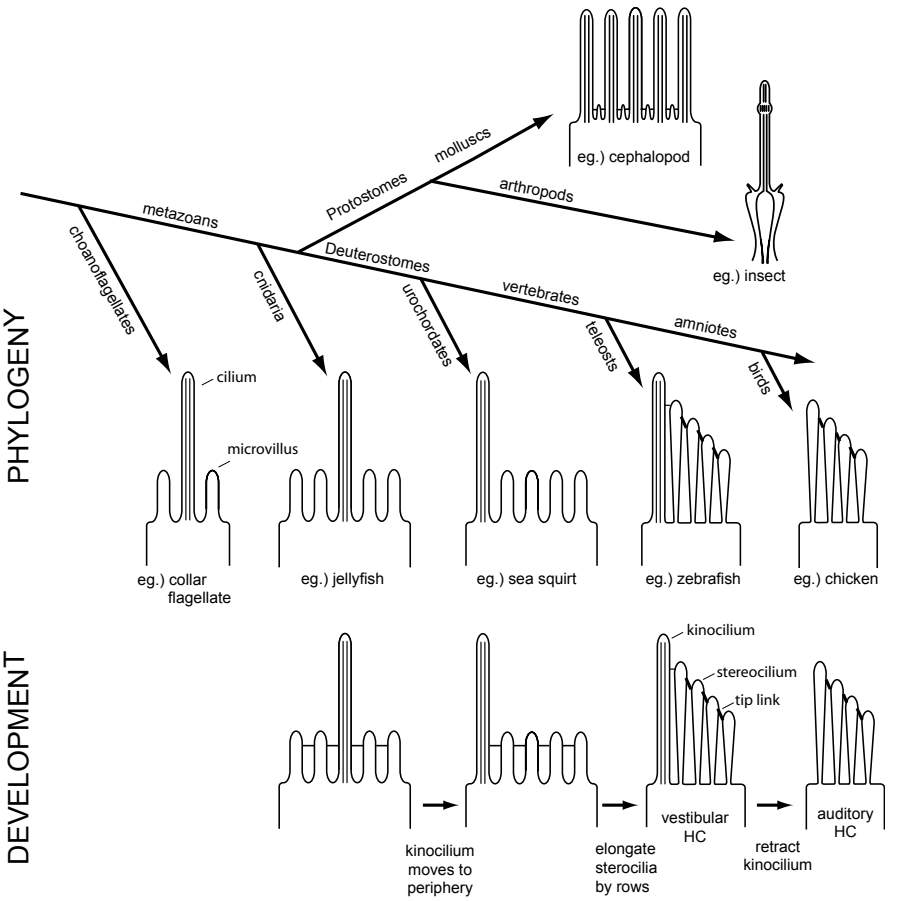

FIGURE 29.2 **Phylogeny *(top row)* and development *(bottom row)* of the apical surfaces of mechanoreceptor cells in representative animals.** Microtubule-based cilia are present in all mechanoreceptors, except for the mature auditory hair cells in birds and mammals, which lose their kinocilium during development. Actin-based microvilli called *stereocilia (stereovilli)* in chordate hair cells are present in many types of mechanoreceptors. The developmental sequence that leads to the maturation of the hair bundles includes a series of steps that elaborate extracellular connectors between apical processes, reorient the kinocilium to the cell periphery, and gradually elongate the staircased arrangement of stereocilia rows (one stereocilium of each row is shown in these schematic cross sections through a bundle). (Modified from Fritzsch et al., 2006; and Goodyear et al., 2006.)

secondary mechanosensory cells with stereocilia (Burighel et al., 2003). Like vertebrate HCs, tunicate sensory cells can synapse with both afferent and efferent fibers, and they are surrounded by auxiliary nonsensory (supporting) cells. Still, Holland (2005) points out that tunicate cells do not have all of the characteristics of a vertebrate HC, and so the controversy about homology remains. In the auditory organs of birds and mammals, the kinocilium is reduced extensively or entirely during HC maturation (see Figure 29.2). Thus, during the evolution of the vertebrate-type HC, the involvement of the cilium has steadily declined until it may only be important for establishing hair-bundle polarity during ontogeny, at least in some auditory organs (described later).

II. NEUROSENSORY CELL FATE SPECIFICATION AND DIFFERENTIATION

A. Neural Fates

One of the earliest cell fates specified in the otocyst is that of the neuroblasts (Rubel and Fritzsch, 2002). These cells leave the otocyst wall, move into the mesenchyme, and gather together to make the *statoacousticganglion (*or *otic ganglion)*. Eventually, the group splits into two ganglia that principally service auditory and vestibular functions. The two classes of neurons can share progenitors among themselves as well as with the cells that remain behind in the otocyst (Satoh and Fekete, 2005). The basic helix–loop–helix (bHLH) genes, *Neurog1* (formerly *Neurogenin1*) and *NeuroD,* are essential for the specification and differentiation, respectively, of certain craniofacial ganglia, including the otic ganglion. In the otic vesicle, these transcription factors are expressed by a subset of epithelial cells around the time of neuroblast delamination. Fritzsch et al. (2000; 2006) have suggested that the regulation of neural fate by *Neurog1* may coincide with the appearance of secondary mechanoreceptors in vertebrates, which must operate in concert with placode-derived postsynaptic neurons. Fibroblast growth factor (FGF) levels can influence the number of neuroblasts in chicken otocysts, and both *Fgf3* and *Fgf10* are expressed in the neurogenic region of the otocyst (Alsina et al., 2004). Because the otic ganglia form in *Fgf10* null mice (Pauley et al., 2003), *Fgf3* and *Fgf10* may have functional redundancy in specifying the neurogenic region. Several other genes may also be involved in specifying otic neuronal identity and/or survival, including *Six1, Eya1,* and *Pax2* (Torres et al., 1996; Zheng et al., 2003; Zou et al., 2004). *Tbx1* is a transcription factor that can repress neural fate in cells that are located adjacent to the *NeuroD* domain. The increase in neuroblasts in *Tbx1* knockout mice occurs at the expense of sensory progenitors (Raft et al., 2004). By contrast, the phenotype of the zebrafish *mindbomb* mutant, which is defective in Notch signaling, shows excessive numbers of both otic neurons and sensory cells (Haddon et al., 1998a). Therefore, it is unclear whether all vertebrates are similar with regard to having a competition among shared "proneurosensory" progenitors that forces a choice between neuronal and sensory fates. One model involves progenitors interacting through Notch-mediated lateral inhibition so that some (but not all) progenitors become neuroblasts, thus allowing others to progress to a "prosensory" state by remaining in the otic epithelium. Although there is limited experimental support for Notch signaling during otic neurogenesis, key components of the signaling pathway appear to be expressed at the appropriate time and place to play such a role (Adam et al., 1998; Alsina et al., 2004; Haddon et al., 1998b; Morrison et al., 1999). After neuroblasts have delaminated from the otic vesicle under the control of the neurogenic genes, *Pou4f1 (Brn3a)* is required for their further differentiation and axon targeting (Huang et al., 2001). *Gata3* is expressed in auditory but not vestibular ganglion neurons (Lawoko-Kerali et al., 2002), leaving open the possibility that this transcription factor is responsible for this binary fate decision.

B. Prosensory Fates

The same domain that gives rise to neuroblasts is at least partially overlapping with prosensory domains. Understanding the genetic regulation of sensory specification in the ear has progressed in recent years, although

many unanswered questions remain. There are mRNAs for several intercellular signaling pathways expressed in regions of the otocyst that will generate sensory organs, including those related to Wnt, Notch, FGF, and bone morphogenetic protein (BMP) signaling. The challenge lies in determining whether these ligands are directly regulating the sensory fate of the expressing cells or if they are used by sensory primordia to organize or regulate cell fates in the surrounding territories. Evidence is beginning to point toward each of these scenarios. It is easier to argue for a cell-autonomous effect in the case of transcription factors, and these will be discussed first; this will be followed by a brief summary of data regarding secreted signaling molecules.

1. Atonal and Sox Transcription Factors

In flies (Drosophila), the specification of sensory organ precursors (SOPs) from which the entire mechanosensory lineage arises was shown to require proneural genes of the bHLH family (Boekhoff-Falk, 2005). *Atonal* is the key proneural gene regulating the development of chordotonal organs, including the Johnston's organ, which is located on the antenna that subserves hearing function in flies. (*Atonal* also regulates proneural function for Drosophila photoreceptors). In Drosophila chordotonal organ development, proneural genes are expressed by an equivalence group of progenitor cells that then uses lateral inhibition to select the SOP cell, the progeny of which develop into primary mechanoreceptor cells, support cells, and glial cells. SOP cells retain *Atonal* expression, whereas other cells in the proneural cluster cease expression and remain epithelial. In other words, the entire chordotonal lineage depends on *Atonal* in the case of the fly's hearing organ (Jarman et al., 1993). When a murine homolog of *Atonal, Atoh1* (formerly *Math1*), was shown to be a deafness gene that was essential for HC fate specification in the mouse (Bermingham et al., 1999), it raised the provocative possibility that we were looking at molecular evidence of a "deep homology" in the evolutionary origin of mechanosensory hearing organs. (*Atoh1* gene expression is not restricted to mechanosensory lineages in vertebrates; it also regulates fates for a select group of neurons outside of the ear).

Within the inner ear, Atoh1's role has been controversial in terms of whether it specifies prosensory progenitors for the entire sensory organ (as opposed to only specifying the HC fate). By definition, a "prosensory" domain contains the progenitors of both HCs and supporting cells, and we expect *Atoh1* to be expressed initially throughout this region if the homology with flies holds up. Indeed, some researchers have noted an appropriately early expression of *Atoh1* in mouse inner ear by *in situ* hybridization, knock-in reporter gene expression, or polymerase chain reaction (Bermingham et al., 1999; Matei et al., 2005; Shailam et al., 1999; Woods et al., 2004). These data support the idea that *Atoh1* is expressed in all progenitors of the prosensory domains and that it only later becomes restricted to HCs. Others have explicitly suggested otherwise, stating that *Atoh1* is exclusively expressed by HCs and that it is not required for establishing a prosensory domain in mice (Chen et al., 2002). As evidence, they describe the normal appearance of a "zone of nonproliferation" in the cochlea of *Atoh1* null mice, which is recognized by a postmitotic patch of cells expressing the cell-cycle inhibitor p27kip1 at the location of the future organ of Corti. In

addition, the expression of a transgene driven by the *Atoh1* promoter is present early and in a small number of cells at prosensory locations, even in the absence of functional *Atoh1* (Fritzsch et al., 2005). Although the prosensory expression and function of Atoh1 remain controversial in the mouse, the bulk of the data indicate that something else must act upstream of Atoh1 to establish a prosensory domain in mice. The missing factor could well be the neural stem-cell–related transcription factor Sox2, at least for the organ of Corti and several vestibular organs (Kiernan et al., 2005b). Two mutant alleles of the *Sox2* locus in mice show the reduction or absence of sensory cells in the cochlea. Significantly, one of these alleles *(Lcc)* lacks both p27kip1 and *Atoh1* expression in the organ of Corti; in other words, it appears to lack the cochlear prosensory domain. As expected in the absence of *Atoh1, Lcc* mice never develop HCs. Whether or not *Sox2* is also required for neuronal fate specification awaits further analysis.

We would like to suggest that mouse development may be a derived state in which Sox2 protein has co-opted the Atoh1 protein's early prosensory function, but that Atoh1's prosensory function may be retained in other vertebrates. In zebrafish, a genome duplication gave rise to two divergent *Atoh1* genes that appear to have subdivided the ancestral Atoh1 protein function. Atoh1b establishes the early prosensory domains while Atoh1a is essential for later HC development (Millimaki et al., 2007). It will be important to determine whether supporting cells are indeed missing when *Atoh1* genes are inactivated in zebrafish and mice, as discussed by Millimaki et al. (2007). If so, this would further solidify the role of the *Atonal* gene family in the specification of mechanosensory lineages in both vertebrates and invertebrates. We eagerly await analyses of additional species to determine the relative role of Atoh1 in the specification of prosensory lineages as compared with HCs before drawing definite conclusions about deep evolutionary homologies underlying the origins of prosensory domains.

2. Notch–Delta Signaling

The involvement of bHLH proneural genes in promoting sensory specification points the finger toward Notch–Delta activation in cells that take on nonsensory fates, because bHLH neurogenic genes are often downregulated by Notch signaling. Indeed, Notch-mediated lateral inhibition of *Atoh1* serves to break a single prosensory domain into two separate patches in the zebrafish inner ear (Millimaki et al., 2007). Likewise, in zebrafish *mindbomb* mutants, which suffer from an inhibition of Notch signaling (Itoh et al., 2003), there is a large increase in the size of the otic sensory patches (Haddon et al., 1998a). A similar phenotype is seen with the deletion of the gene encoding the Notch ligand, DeltaA, in zebrafish (Riley et al., 1999). Also, the targeted deletion of the gene encoding the Notch1 receptor in the mouse inner ear shows an apparent increase in the size of the sensory domain in the cochlea (Kiernan et al., 2005a). Altogether, these loss-of-function studies support a role for Notch-mediated lateral inhibition in regulating the size and spatial distribution of the prosensory domain: too little Notch signaling generates too many sensory cells. One would therefore predict that too much Notch signaling would result in a reduction of sensory cells. In striking contrast to this prediction, gain-of-function for Notch–Delta signaling in the chicken promotes rather than inhibits the prosensory fate (Adam et al., 1998). Specifically, ectopic

sensory patches can be induced in the nonsensory parts of the cochlear duct by forcing the expression of *Notch1*^{ICD}, encoding the intracellular domain of the Notch receptor that can directly regulate gene transcription in the absence of a ligand (Daudet and Lewis, 2005). In addition, loss-of-function mutations of *Jag1,* a Notch ligand gene expressed in sensory primordia, display a near absence of vestibular sensory organs and reduced numbers of sensory cells in the cochlea (Brooker et al., 2006; Kiernan et al., 2001, 2006). The prosensory domain for the organ of Corti is significantly reduced in size. These seemingly paradoxical data may be resolved if Jag1 interacts with a Notch receptor other than Notch1 to promote prosensory fates, whereas different ligands (Jag2 and Delta1) perform the more classic role of laterally inhibiting sensory fates through Notch1 (discussed further in the section on Sensory Cell Types).

3. Wnt Signaling

The "prosensory" phenotype induced by the activation of Notch is remarkably similar to that seen after manipulation of the Wnt/β-catenin signaling pathway (Daudet and Lewis, 2005; Stevens et al., 2003). Sensory patches are induced directly and cell-autonomously after the forced misexpression of an activated form of the Wnt intracellular signaling component, β-catenin. However, not all parts of the ear anlage appear to be sensory competent in this assay, because dorsal otic cells are not responsive. It may be that only cells residing within the "prosensory" or "sensory-competent" domain in the ventral ear can be forced to assume a sensory fate through ectopic Wnt signaling. Messages for a variety of Wnt ligands and their Frizzled receptors are expressed in the developing inner ear in chickens (Stevens et al., 2003; Sienknecht and Fekete, unpublished), although the identity of endogenous ligand(s) that may mediate sensory fate specification remains elusive. Data from TOPgal reporter mice, which are designed to reveal cells that are responding to β-catenin–mediated signaling, are puzzling: activity is found in the dorsal (primarily nonsensory) otocyst from embryonic days 8.5 through 11.5 as expected by the dorsalizing role of Wnts (discussed later), but this location is inconsistent with Wnt-mediated prosensory function (Riccomagno et al., 2005). Nonetheless, by embryonic day 12.5 in the mouse, the utricular macula and the cristae are also positive in TOPgal reporter mice (Fekete and Wu, unpublished), which suggests that dorsally located sensory organs are indeed responding to a Wnt/β-catenin signal with an unclear function. However, this reactivity appears too late to be directly responsible for sensory organ specification.

4. Fibroblast Growth Factor Signaling

Fgf10 is expressed in sensory primordia, and FGF receptors (FGFR) 1 and 2 are required for proper sensory development (Pauley et al., 2003; Pirvola et al., 2000; 2002). In fact, loss-of-function of *Fgfr1* in the mouse ear generates a phenotype that bears a striking similarity to a weak *Sox2* allele, *yellow submarine (Ysb)*; both form small islands of sensory cells scattered through larger regions that are devoid of HCs (Kiernan et al., 2005b). *Fgf3* expression also overlaps partially with *Fgf10,* and, together, these molecules and their receptors are likely to be important in the development of both sensory and nonsensory structures in the inner ear (Fritzsch et al., 2006).

The presence of FGFs in sensory primordia also influences the development of adjacent nonsensory structures. In chicken embryos, otocyst infection with retroviruses or the implantation of beads soaked in FGFs were used to force excessive or ectopic FGF signaling, whereas the delivery of beads soaked in FGF inhibitors reduced FGF signaling. Results from these manipulations, in combination with fate-mapping data, led to a model whereby FGFs emanating from incipient cristae enhanced the outgrowth of the associated semicircular canals by upregulating *Bmp2* in canal precursors located just beyond the prosensory domain (Chang et al., 2004).

5. Bone Morphogenetic Protein Signaling

One of the earliest markers of incipient sensory domains is *Bmp4*. There are two foci of *Bmp4* expression at the anterior and posterior poles of the otocyst in nearly every species in which it has been examined, including mouse, chicken, frog, and zebrafish. Although the appropriate fate-mapping studies have not been performed, these foci are believed to give rise to the earliest sensory primordia, which may be the anterior and posterior maculae (zebrafish) or the cristae (mouse and chicken). In the chicken, *Bmp4* expression eventually marks the location of each sensory primordium. It has proven difficult to move beyond using *Bmp4* mRNA as a sensory marker to understanding its functional role in ear development, in part because BMPs are so crucial during the early events of embryogenesis that genetic knockouts or blocking studies generally cause severe phenotypes, including embryonic lethality. Instead, informative data have come primarily from experimental embryology of the developing ear, such as implanting beads soaked in BMP antagonists or injecting viruses encoding *Bmps* or the BMP antagonist, noggin (Chang et al., 1999; Gerlach et al., 2000). Although the results show a BMP-dependent process for canal formation, a key observation was that sensory organ differentiation remained intact in these in vivo experiments. By contrast, one study using cultured chicken otocysts indicated a prosensory role for BMP, because its inhibition by antagonists such as noggin or soluble BMP receptors decreased HC numbers, whereas excess BMP4 increased HC numbers (Li et al., 2005). However, another study came to the opposite conclusion after providing more convincing data showing that BMP treatment reduced the number of proliferating progenitors and in this way reduced the number of differentiated HCs (Pujades et al., 2006). More information using conditional gene targeting in mice will be helpful to confirm these conclusions and to determine how directly BMPs may control proliferation and/or differentiation of the sensory progenitors.

C. Sensory Cell Types

Sensory organs of the inner ear generate HCs and supporting cells as their two major cell types, and these arise from a common progenitor (Fekete et al., 1998). Notch signaling is intimately involved in the HC-supporting cell fate decision as evidenced by genetic mutations in mice or in ovo experimental manipulations in chickens. This is the third time that we have seen Notch signaling play a role in the development of the inner ear (the first two being during neurogenesis and prosensory specification). The Notch signaling pathway may have two independent roles in specifying HCs and supporting cells, as indicated by Figure 29.3 (reviewed by Goodyear et al., 2006). First, there is

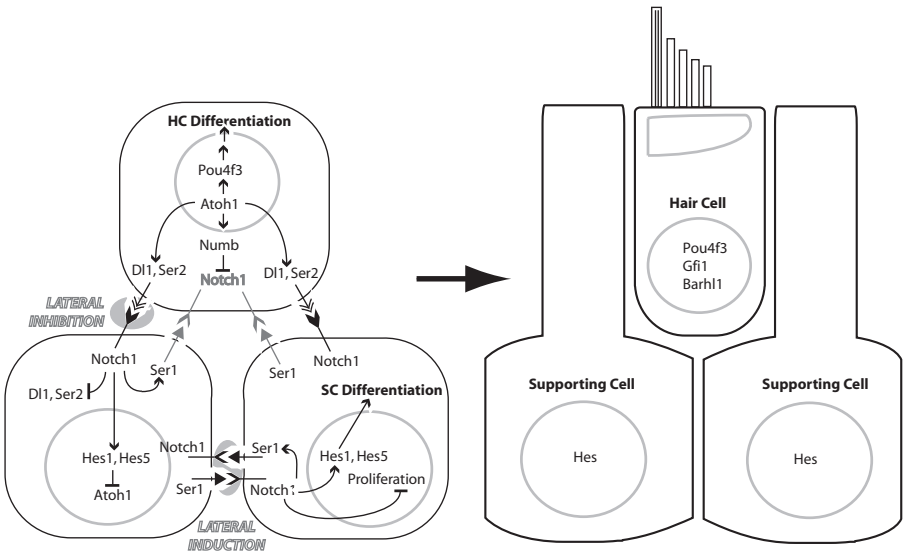

FIGURE 29.3 The Notch signaling pathway specifies hair cell fates through lateral inhibition, and it may promote the supporting cell fate through lateral induction. Different Notch ligands appear to be responsible for these two distinct processes. Key basic helix–loop–helix genes that are either activated or repressed as a result of Notch signaling are shown, including *Hes1*, *Hes5*, and *Atoh1*. (Modified from Goodyear et al., 2006.)

its more classical role in what is called *lateral inhibition,* during which, initially, all cells of the sensory primordium are members of an equivalence group with the potential to become either an HC or a supporting cell. Through a so-far unknown process, an individual cell begins to acquire the primary HC fate, and it will inhibit the cells that contact it, relegating them to the secondary fate of supporting cell. Lateral inhibition from a nascent HC to a nascent supporting cell is likely mediated by two Notch ligands, Delta1 and Serrate2 (also called Jagged2). The nascent HC itself is rendered insensitive to Notch-mediated inhibition, because it begins to express Numb, an intracellular protein that ubiquitinates membrane-bound Notch and causes its degradation (McGill and McGlade, 2003). Nascent supporting cells become less able to laterally inhibit nascent HCs in return, because activation of their Notch1 receptors downregulates the expression of *Delta1* and *Serrate2*. A second Notch-mediated interaction occurs between supporting cells. Here, Notch is activated by a different ligand, Serrate1 (Jagged1), to control genes that are presumed to potentiate the supporting cell fate. This process has been called *lateral induction* (Eddison et al., 2000). There is now evidence that inhibiting Notch signaling not only generates more HCs (as predicted by a lateral inhibitory model) but that it also stimulates supporting cell division (Kiernan et al., 2005a). Using Notch-mediated induction to reduce proliferation among the supporting cell pool may be a necessary prerequisite for these progenitors to progress along a differentiation pathway.

As mentioned previously, HCs have an essential requirement for *Atoh1* for their specification (Bermingham et al., 1999). What is less clear is whether Atoh1 acts upstream or downstream of the Notch-mediated selection of cell fates from among a common pool of sensory progenitors, particularly in mice. If *Atoh1* is expressed throughout the prosensory domain (as described earlier)

but is only required for HC specification, then the sole purpose of Notch lateral inhibition may be to prevent all potential sensory cells from acquiring an Atoh1-mediated HC fate, thereby leaving some to differentiate as supporting cells. In zebrafish, a total conversion of the sensory primordia into HCs indeed accompanies reduced Notch signaling, as predicted by a lateral inhibition model (Haddon et al., 1999). This contrasts with findings in mouse models, in which the knockout of *Notch1* fails to completely eliminate supporting cells at the expense of HCs (Kiernan et al., 2005a). The loss of supporting cells in the *Atoh1* mutant is thought to be a secondary consequence of HC loss (Bermingham et al., 1999) rather than a consequence of supporting cells requiring the gene at an earlier prosensory stage. Furthermore, the ectopic expression of *Atoh1* induced ectopic HCs, which in turn induced the formation of supporting cell fates without requiring an intermediate upregulation of *Atoh1* in the responding cells (Woods et al., 2004). In other words, HCs but not supporting cells require *Atoh1* for cell fate specification.

Despite some uncertainties about Atoh1's exclusivity for mammalian HCs as compared with prosensory cells, the subsequent process of HC survival and differentiation requires the genes for at least three HC-specific transcription factors, *Pou4f3, Gfi1,* and *Barhl1* (Erkman et al., 1996; Hertzano et al., 2004; Li et al., 2002; Wallis et al., 2003; Xiang et al., 1997). The absence of each of these genes eventually leads to HC death at progressively later stages of HC differentiation. *Gfi1* expression is positively regulated by *Pou4f3,* and it underlies a form of human deafness (Hertzano et al., 2004).

D. Planar Cell Polarity of Hair Bundles

The systematic orientation of stereociliary bundles within hair-cell–bearing sensory organs is an outstanding example of *planar cell polarity* (PCP), a term that refers to cellular polarity along the surface of an epithelium. The morphogenesis of the hair bundle has been well described (reviewed by Goodyear et al., 2006), and it is shown schematically in Figure 29.2. The kinocilium emerges from the center of the HC's apical surface, and this is followed by the appearance of elongated microvilli around it. Some of the microvilli are nascent stereocilia; they become tethered to each other by extracellular bridges called *lateral links*. The kinocilium then moves centrifugally toward one side of the developing bundle. There is a systematic elongation of the stereocilia by rows, with stereociliary height increasing earlier on the basis of proximity to the kinocilium. At the same time, the uppermost lateral links connecting the stereocilia appear to stretch to an oblique angle as they span the distance from the tip of a shorter stereocilium to the side of the adjacent taller one. In other words, the distal lateral links may differentiate into *tip links*. The tip links are an essential component of mechanotransduction; they are presumed to hold the mechanotransduction channels in a state of tension so that their open probability is raised in response to miniscule movements of the bundle. Stereociliary numbers are culled through selective resorption as the bundle matures. The initial bundle asymmetry assumed when the kinocilium moves to the periphery often needs further refinement or reorientation so that adjacent HCs become aligned with a global polarity across the sensory field. In the auditory HCs of birds and mammals, the kinocilium generally degenerates as a final step in hair bundle maturation.

The process of bundle development bears a striking similarity to evolutionary changes in mechanosensory cell bundle morphology, which becomes obvious when comparing representative metazoan species (see Figure 29.2). Considerably more data from stem group species are needed to determine whether this truly represents a case of "ontogeny recapitulating phylogeny."

Bundle orientation in the organ of Corti appears particularly vulnerable to genetic mutations that disrupt patterning and cell fate specification, including that of the Notch signaling pathway (Kiernan et al., 2005a). Even more intriguing in the context of evolutionary conservation are reports that local deviations in bundle orientation arise from mutations in vertebrate orthologs of the Drosophila PCP pathway (Kelley, 2006). Examples include *Vangl2, Dishevelled1/2,* and the atypical cadherin *Celsr2* that are orthologous to Drosophila *strabismus, dishevelled,* and *flamingo,* respectively (Curtin et al., 2003; Montcouquiol et al., 2003; Wang et al., 2005). There are several other genes with mutant phenotypes that perturb bundle orientation but that are not known to be related to the fly PCP pathway. These include *scribble,* which interacts genetically with *Vangl2* (Montcouquiol et al., 2003), and *PTK7,* encoding a receptor tyrosine kinase (Lu et al., 2004). The PCP pathway in flies involves a Frizzled receptor, and two different Frizzled genes have been implicated in PCP in HCs (Wang et al., 2006). Although in flies there is no requirement for wingless (the Frizzled ligand), there is a link between Wnt7a and cochlear hair bundle polarity in mice (Dabdoub et al., 2003; Dabdoub and Kelley, 2005). All of the mutants and protein perturbations cited above yield local bundle orientation defects that worsen from the first to the third row of outer HCs. Inner HCs and additional outer HC rows are generally not affected unless the phenotype is enhanced by double gene knockouts (Wang et al., 2005, 2006). Interestingly, the centrifugal migration of the kinocilium and the staircased elongation of stereocilia, which are processes that underlie bundle asymmetry, progress normally in these mutant mice. Lewis and Davies (2002) predicted that there must be three tiers of control for polarizing HCs: internal (bundle asymmetry), local (neighboring HCs orient similarly), and global (entire fields of HCs). The studies reported to date are most consistent with discovering genes that influence polarity at the local levels; this leaves regulation at the intracellular and global levels completely unresolved.

III. PATTERN FORMATION IN THE INNER EAR

Having considered the specification of neural and sensory fates at the level of individual cells or small groups of cells, we now turn our attention to how these cells are positioned appropriately in their environments. Specifically, we seek to understand how the otic ectoderm arises at its precise position with respect to the overall body plan and how each sensory patch arises at a specific location with respect to the overall organization of the inner ear. To optimally couple mechanical fluid movement with hair bundle stimulation, the development of the fluid ducts, chambers, and extracellular specializations overlying sensory organs must somehow be spatially coordinated with the development of the HCs, and there is now evidence that this is indeed likely. We begin with a discussion of how the otic placode comes to lie adjacent to rhombomeres 4 through 6, which is the problem of otic induction. We follow this with a brief discussion of the establishment of the major axes of the ear

(dorsal–ventral, medial–lateral, and anterior–posterior), because this is essential for subsequent pattern formation. We end with a presentation of a few regionally expressed genes as examples of how the spatial restriction of sensory foci, in combination with more broadly expressed "selector" genes in otic subcompartments, may underlie the specification of different ear parts and thereby initiate the complex process of inner ear morphogenesis.

A. Otic Placode Induction

For nearly 100 years, developmental biologists have been intrigued by the process of otic induction, and they have sought to discover which tissues and, more recently, which molecules induce the otic placode. Early embryologists used the overt morphologic appearance of the otocyst itself as the principle criterion to score for induction after performing various types of tissue manipulations. Tissue extirpations, transplantations, and rotations were typically employed to reveal the source(s), distribution, and timing of otic-inducing factors. It has recently become possible to detect the onset of otic specification at earlier stages by using molecular markers rather than morphogenesis as definitive evidence of otic fate. Examples of such markers include transcription factors of the *Pax* gene family. The discovery of new molecular markers coincided with an active pursuit of the molecular nature of the otic inducers. Members of the FGF family have emerged as leading contenders for otic inducers in all vertebrate model organisms studied to date. The sources of FGF include the hindbrain and the mesendoderm in the vicinity of the heart primordium. There are two striking aspects to the story. First, there is considerable redundancy, with more than one FGF typically acting as an otic inducer within a single species. Second, the exact members of the FGF family used as otic inducers varies for different species, but *Fgf3* is always included. Also linked with otic induction are *Fgf8, Fgf10,* and/or *Fgf19* in various species. A requirement for Wnt signaling in otic specification is not as well conserved among vertebrates (Ladher et al., 2000; Ohyama et al., 2006; Phillips et al., 2004). The reader is directed to several excellent reviews that have merged the classic and modern literature regarding otic induction (Groves, 2005; Noramly and Grainger, 2002).

Studies in zebrafish have described a complex network of transcriptional activation that is downstream of *Fgf3*- and *Fgf8*-mediated inductive signaling from the hindbrain (Maroon et al., 2002; reviewed by Riley and Phillips, 2003; Whitfield et al., 2002). In parallel, *Foxi1* acts in the early placode and *Dlx3b/4b* acts a little later to endow the ear tissue with competence to upregulate *Pax* and other otic genes. In zebrafish, *Foxi1* is one of the earliest otic genes; without it, the ear is severely hypomorphic (Solomon et al., 2003). *Sox9* is also involved in early ear development in Xenopus (Saint-Germain et al., 2004). A model has emerged for zebrafish otic induction in which two parallel pathways provide genetic redundancy: FGF arising externally from the hindbrain and *Foxi1* acting internally to give preplacodal cells competence to respond to this inducer (Hans et al., 2004; Solomon et al., 2004). After it has been initiated, the process is further maintained by a *Dlx–Pax2* pathway.

Foxi1 is unlikely to play such a pivotal role in the mouse ear, because its expression in the otocyst is too late and too restricted, which fits with the late onset of inner ear defects in *Foxi1* mutant mice (Hulander et al., 2003;

Ohyama and Groves, 2004). Although other *Foxi* and *Foxg* family members are expressed in the placodal and otic ectoderm of the mouse, none has yet been shown to have a similar function to zebrafish *Foxi1* in terms of otic competence (Ohyama and Groves, 2004; Pauley et al., 2006). Likewise, although members of the *Sox, Six, Dlx, Eya,* and *Pax* gene families are impressive as early markers of the otic placode in several species, their absence in genetic null mice does not prevent the induction of the otic placode (Groves, 2005). However, although they are not required for otic induction *per se*, mutations in these transcription factors show them to be essential for proper ear morphogenesis in mice and zebrafish (reviewed by Groves, 2005; Riley and Phillips, 2003). Members of a *Pax/Six/Eya/Dach* gene network are active in many placodal derivatives, including other sensory-associated tissues, such as the lens and olfactory placodes (Baker and Bronner-Fraser, 2001). In summary, a subset of the molecular signals for otic induction (FGFs) and much of the downstream genetic program for early otic development (via a *Pax–Six–Eya* network) appear to be conserved through vertebrate evolution.

B. Axis Specification

The otic placode invaginates into the underlying mesoderm, first forming a cup and eventually pinching off to become a vesicle (except in zebrafish, where a cavitation process occurs). The medial half of the otic vesicle (otocyst) comes into close contact with the hindbrain in the region of rhombomeres 4 through 6. There has long been evidence that otocyst patterning is influenced by the flanking hindbrain (Fritzsch et al., 1998). Recent progress within the last 4 years has identified some of the secreted factors involved.

Dorsalization of the mouse ear is mediated through Wnt/β-catenin signaling, specifically *Wnt1* and *Wnt3a,* which are expressed by the dorsal neural tube (Riccomagno et al., 2005). Ventralization of the mouse ear requires Sonic hedgehog protein that originates from the notochord or the ventral floor plate of the neural tube (Riccomagno et al., 2002; Bok et al., 2005). A number of genes are likely to be directly or indirectly responsive to these hindbrain signals, including *Gbx2, Wnt2b,* and *Dlx5/6* in the dorsal otocyst and *Otx2* in the ventral otocyst (Choo et al., 2006; Lin et al., 2005).

The hindbrain also influences the medial–lateral expression of genes within the otocyst (Brigande et al., 2000). For example, in several different mouse mutants in which hindbrain development is abnormal, medial otic genes are downregulated or shifted more laterally. In other mutants, laterally expressed genes expand into the medial territory, particularly when the otocyst is located too far from the hindbrain. Finally, the manipulation of either the hindbrain or the otocyst reveals that normal juxtaposition of the two is required to maintain medial gene expression in the chicken otocyst (Giraldez, 1998; Hutson et al., 1999). We still need to learn more about the identity of the molecular signals arising from the hindbrain that regulate gene expression along the medial–lateral axis of the inner ear.

Specification of the anterior–posterior (AP) axis of the otocyst is also not well understood at a molecular level. The AP axis of the sensory organs is not fixed until after the 16-somite stage in chickens, and yet manipulative reversal of the AP axis of the hindbrain between the 10- and 13-somite stages fails to reverse AP axial specification in the otocyst (Bok et al., 2005; Wu et al., 1998). The implication is that AP patterning signals for the inner ear do not

originate from the hindbrain. In the zebrafish, pattern duplications along the AP axis can occur after perturbations in Hedgehog signaling (Hammond et al., 2003). This is a curious departure from Sonic hedgehog's role in ventralizing the inner ear in the mouse and chicken, although new evidence suggests that the repression of Hedgehog signaling may indeed be required to establish dorsolateral otic fates in zebrafish (Hammond, van Eaden, and Whitfield, presented at the 2006 International Zebrafish Meeting, Madison, WI). One possible explanation for the species differences is that the specific ventralizing effects of Hedgehog proteins may be restricted to vertebrates that have evolved a cochlear duct (i.e., the land vertebrates).

C. Regionalization of the Otocyst

Although we have yet to enjoy a comprehensive molecular model for understanding the regionalization of the inner ear, great strides have been made in revealing a plethora of transcription factors that are required for generating different structural parts, such as canals, utricles, saccules, cochlear ducts, and so on. Much of this knowledge arises from analyzing null phenotypes in transgenic mice (Cantos et al., 2000; Fekete, 1999). One model suggests that the ear becomes subdivided into developmental compartments after axial specification and that a set of so-called "selector genes" acts within these compartments to specify each ear part (Brigande et al., 2000; Fekete, 1996; Kiernan et al., 1997). This would be akin to the combinatorial Hox code for specifying segments of the insect body or the vertebrate hindbrain (see Chapter 9). In the inner ear, we are not yet able to decipher such a code or even to confirm that it exists. Moreover, we should be wary of oversimplifying ear regionalization, because there appears to be an important interplay between the specification of sensory primordia, which occurs very early, and the later process of morphogenesis of ducts and canals. The best example is the role of the cristae in the induction of canal morphogenesis (discussed later).

The specification of inner ear parts by potential "selector genes" is complex, and the story is still unfolding (Cantos et al., 2000; Fekete, 1999; Fritzsch et al., 2006; Riley and Phillips, 2003). By way of example, we summarize the data for a few transcription factor genes with null phenotypes that have been especially well documented in the literature. The cochlear duct, often in combination with the saccule, shows severe hypomorphism in null mutants of *Pax2* and *Otx1* (Burton et al., 2004; Morsli et al., 1999). The disruption of one or more semicircular canals is observed in knockouts of *Hmx* and *Otx* genes (Morsli et al., 1999; Wang et al., 2004). The development of dorsal otic structures, including the endolymphatic apparatus and the semicircular canals, are affected by null mutations in *Dlx5, Gbx2,* and *Gata3* (Acampora et al., 1999; Lilleväli et al., 2006; Lin et al., 2005).

In addition to numerous transcription factors, several secreted factors are also important for the regionalization of the ear. Progress in this area has taken advantage of the accessibility of the chicken embryo to focally manipulate signaling molecules, such as FGFs, retinoic acid (RA), and BMPs. For example, pathologic effects can be seen in the inner ear in response to both vitamin A deficiency and the excess application of RA. Retinoids are likely to influence ear morphogenesis at several different points, beginning with their effects on the hindbrain, which can then indirectly influence the otocyst (Kil et al., 2005). In addition, a number of components in the RA signaling pathway are expressed

directly in the otocyst or the surrounding periotic mesenchyme, thereby supporting a more direct role in these tissues as the ear develops (Romand, 2006). Semicircular canal morphogenesis appears to be especially sensitive to exposure to excess RA (Choo et al., 1998), to the downregulation of FGFs (Chang et al., 2004), and to treatment with BMP inhibitors such as noggin (Chang et al., 1999; Gerlach et al., 2000). Overall, the data support the idea that the local production of secreted factors in the sensory primordia act in a paracrine fashion to regulate the outgrowth of nonsensory structures. Several of these signaling pathways, including those for RA and BMPs, also affect the periotic capsule, thereby revealing the molecular basis of the epithelial–mesenchymal interactions that have long been recognized in this system (Romand, 2006).

Programmed cell death occurs in various locations, which suggests that it is a key component of inner ear morphogenesis. Manipulation in chicken embryos and the analysis of mouse mutants are beginning to shed light on the process, including the discovery that the apoptotic protease activating factor 1, Apaf1, plays a crucial role in the inner ear (Cecconi et al., 2004; Fekete et al., 1997; Leon et al., 2004).

IV. PHYLOGENETIC AND EVOLUTIONARY CONSIDERATIONS

A. Phylogeny of Mechanoreceptive Organs

The hypothesis that *Atonal*-like gene expression was coincident with the origin of mechanoreceptors, thereby forcing the idea of a common ancestral mechanoreceptor, is still under debate. However, the data in favor of this hypothesis continue to accumulate as molecular development is analyzed in a broader range of organs and organisms. There is a growing list of genes other than *Atonal* homologs that are also shared between the hearing organs of flies and vertebrates, including *spalt/SAL* genes, *distalless/Dlx* genes, *crinkled/Myosin-VIIa,* and transient receptor potential channels, to name a few (reviewed by Boekhoff-Falk, 2005). The parallels have now become impossible to ignore.

When considering these issues, we wish to focus attention not so much on the evolutionary origin of mechanoreceptors *per se* but rather on the proneurosensory epithelial patch from which they derive. To date, we have unambiguous lineage information only for Drosophila and chicken. In flies, the entire chordotonal lineage arises from an *Atonal*-expressing ectodermal patch that gives rise to the primary (neuronal) mechanoreceptors and their associated cells. *Atonal* thus serves as a proneural gene for chordotonal organs, including the Johnston's organ, which is specialized for hearing. Likewise, in vertebrates, the neurons, sensory organs, and associated nonsensory epithelial cells of the inner ear all arise from an ectodermal otic placode. Lineage analysis confirms that common progenitors for these various cell types indeed exist (Satoh and Fekete, 2005). *Atoh1b* is expressed in the zebrafish prosensory patch of the inner ear, probably overlapping with the neurogenic region. If this represents an ancestral vertebrate condition, then it would extend the parallels with the fly to the earliest stages of mechanosensory organ specification. Perhaps it is here, at the proneurosensory step, that the common ancestor of vertebrates and invertebrates evolved a dependence on an *Atonal* homolog to establish a mechanosensory lineage. It is then relatively easy to imagine that organs using either primary or secondary types of receptor configurations could both have evolved from an epithelial patch with the competence to generate

either neurons or epithelial mechanoreceptors. In fact, all three cell types of interest (neurons, primary receptors, and secondary receptors) are derived from the preplacodal field in vertebrates, although primary receptors derive only from the olfactory placode in extant species (Schlosser, 2005). This raises an intriguing question: is there also a shared lineage in derived cephalopods between its secondary mechanoreceptor and the neuron that innervates it? Might the new field of "evo–devo" contribute an answer to the question of whether the secondary mechanoreceptors found in cephalopod molluscs and vertebrates are indeed a case of convergent evolution, or are they far more similar than we suspected with regard to their developmental and evolutionary origins?

B. Evolution of the Vertebrate Inner Ear

A comparison of vertebrate inner ears reveals considerable diversity in the number and arrangement of sensory organs and the chambers that house them. A vestibular labyrinth can be traced back to the earliest craniates, the fossilized ostracoderms (agnathes), which possessed two semicircular canals (each presumably associated with a crista) and a central vestibule (perhaps with at least one macula). Living agnathes (hagfish, lamprey) have a single macular sense organ (the "macula communis"), two cristae, and associated canals (although in modern hagfish there is only one canal). The jawed vertebrates possess three canals with cristae and several separate maculae (Ladich and Popper, 2004). The vertebrate ear typically contains a minimum of two major maculae (the saccular and utricular maculae), where the HCs are covered by an otolithic membrane. Additionally, a variable number of generally smaller otolithic sensory organs (e.g., the maculae lagena and neglecta) can be found in a subset of species, whereas only land vertebrates have a dedicated hearing organ located in the cochlear duct.

A great range of variations in the organization of inner ear sensory epithelia is realized among different groups of amphibians. It is usually argued that the single macula communis has been repeatedly and perhaps independently subdivided during vertebrate evolution (Fritzsch et al., 2002; Fritzsch et al., 2006). The embryonic development may reflect this where the prosensory primordium begins as a single patch which then segregates into different sensory organs (Smotherman and Narins, 2004). However, molecular markers such as *Bmp4* reveal not one but two sensory primordia arising independently at the anterior and posterior poles of the developing otocyst in zebrafish, Xenopus, chickens, and mice. Also compelling is the finding that the single macula communis of lamprey in fact "originates as two foci of hair cells" (Hammond and Whitfield, 2006) that subsequently fuse. A careful analysis of sensory organ development in different species may reveal whether newly evolved organs arose by splitting off of an existing organ, by the *de novo* appearance of new foci from within a contiguous field of prosensory cells, or both.

Elegant comparative work from the Whitfield lab indicates that the *Otx1* gene seems to be responsible for the evolutionary emergence of the third semicircular canal on the lateral wall of the otocyst (Hammond and Whitfield, 2006). The zebrafish inner ear, which normally has three canals, comes to resemble the two-canaled lamprey ear after a knockdown of *Otx1*. Furthermore, like the lamprey, the separate anterior and posterior maculae fuse together in the absence of *Otx1*. This phenotype is remarkably similar to that of the *Otx1* null mouse, which lacks the horizontal canal and shows fusion of

the utricular and saccular maculae (Morsli et al., 1999). These data suggest that the evolution of the vertebrate ear was strongly influenced by *Otx1*.

C. Evolution of Dedicated Hearing Organs

Later evolution of the vertebrate inner ear is characterized by the invention of hearing capabilities, initially by expanding the function of otolith-bearing vestibular organs (Ladich and Popper, 2004). For example, fish do not appear to have evolved a unique sensory epithelium that is entirely dedicated to acoustic function. Instead, their diverse otolith-bearing "vestibular" maculae mediate the senses of linear acceleration and/or hearing. The bandwidth of their hearing can approach that of many amphibians and birds (Ladich and Popper, 2004; Popper and Fay, 1999). With the exception of some known hearing-specialist teleost fish species, in which the utricle is highly derived, the enormous structural diversity of the saccule and occasionally the lagena is thought to be linked to both vestibular and auditory function.

Auditory co-option of vestibular maculae occurs not only in fish but also in amphibians, in which the saccule serves primarily as a specialized terrestrial seismic detector for sensing vibration and sound; it is, therefore, an essential part of the auditory system (Smotherman and Narins, 2004). Modern amphibians such as frogs typically use two sensory organs for auditory detection: the amphibian papilla and the basilar papilla. Whereas the basilar papilla of amphibians appears to be a simple resonant structure (Meenderink et al., 2005), the amphibian papilla is tonotopically organized and has extended sensitivity. In much the same way, a group of less-derived amphibia, the caecilians, show modifications of the saccule to enhance its capabilities as an auditory receptor. Even in mammals, some vestibular afferents show responsiveness to sound (McCue and Guinan, 1995).

The first otic epithelium in vertebrate evolution that responded exclusively to sound is probably the amniote (land vertebrate) basilar papilla (Manley and Ladher, 2007). Although the origin of this auditory organ is not yet clear, it can be traced back with certainty to stem reptiles, which implies that it has evolved over a period of more than 300 million years (Manley and Clack, 2004). Among living land vertebrates, there can be found a remarkable variety of this hearing organ, which has a structure that differs characteristically for each taxonomic group. There are evolutionary trends in the morphology and physiology of the amniote auditory receptor organ that lead to the phenomenon of parallel evolution of a variety of hearing organs, especially after the invention of middle ears during the Triassic period. These trends are as follows: (1) the elongation of the auditory epithelium, which results in an increase of micromechanical tuning options and the space devoted to each octave; (2) structural gradients across the basilar papilla; and (3) the specialization of HC functions (Manley and Clack, 2004).

In our view, developmental biology may contribute to a better understanding of how different vestibular organs evolved and how some of them came to subserve hearing. For example, the question of which macula eventually gave rise to the basilar papilla of amniotes (including the organ of Corti of mammals) might be clarified if the cochlea and another organ, such as the saccular macula, were both shown to arise from a unique prosensory subdomain and/or to share unique genetic regulators at an early stage of their specification. Likewise,

evolutionary relationships might emerge by exploring the developmental sequence of sensory organ appearance in a range of vertebrates, such as various amphibians, lizards, and crocodiles. When combined with physiological evidence showing which organs mediate hearing, these studies could help to clarify what is otherwise a dizzying array of inner ear diversity.

SUMMARY

- The vertebrate inner ear arises from the otic placode, invaginates to form a fluid-filled epithelial ball (the otocyst), and "morphs" into its major compartments, which are involved in balance (semicircular canals, utricle, saccule) and hearing (usually the cochlea). Some species have modified vestibular organs that subserve hearing.
- Pattern formation within the epithelium of the inner ear involves a sequence of events that includes otic placode induction by FGF-mediated signaling; the dorsal–ventral axis specification through Wnt (dorsal) and Sonic hedgehog (ventral) signals; and the regionalization of the ear that is influenced by a large number of transcription factors and signaling molecules to generate sensory and nonsensory components.
- Animal models showing defects in inner ear development have provided important clues about the underlying causes of human deafness, including defects in inner ear morphogenesis, mechanosensory hair cell differentiation, and the regulation of the special ionic environment of inner ear fluids.
- Notch signaling plays an important role in cell fate specification at least three different times during inner ear development: during neurogenesis, during prosensory organogenesis, and during the making of the HC/supporting cell fate decision.
- The development of the hearing organs of vertebrate and invertebrate model organisms both require *Atonal* homologs. The two systems also share other genes that are involved in mechanosensory cell function, and this leads to speculation that there may be a deep evolutionary homology in the origin of mechanoreceptors.

GLOSSARY

Basilar papilla
A hearing organ in the vertebrate inner ear that is found in birds, reptiles, and some amphibians and that is homologous to the organ of Corti in the mammalian cochlea.

Crista
A mound-like sensory organ in the vertebrate inner ear, residing in a chamber called the *ampulla,* that is associated with the semicircular canal and that senses angular acceleration.

Hair cells
Mechanoreceptor cells that are characterized by apical specializations in the form of a staircased arrangement of modified cilia called *stereocilia* that are used to detect fluid movements.

Macula
A patch-like sensory organ in the vertebrate inner ear that senses gravity. In some fish and amphibians, it can be specialized as a hearing sensor.

Mechanoreceptor
A sensory cell that converts mechanical energy into electrical signals that can ultimately be used for the neurochemical transmission of information to the central nervous system.

Otocyst
An epithelial ball of cells lying on each side of the hindbrain that develops into the vertebrate inner ear.

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30 CRANIOFACIAL FORMATION AND CONGENITAL DEFECTS

S. A. BRUGMANN and J. A. HELMS

Department of Plastic and Reconstructive Surgery, Stanford University, Palo Alto, CA

INTRODUCTION

> "What is a face, really? Its own photo? Its make-up? Or is it a face as painted by such or such painter? ... Doesn't everyone look at himself in his own particular way? Deformations simply do not exist."
>
> *Pablo Picasso*

Poets and artists describe the face as a mirror of the soul. To those interested in craniofacial biology, the face is also a reflection of remarkable structural diversity that can exist even within a single species. Six billion humans decorate the earth, each of their faces exceptional in its own way. Despite this inherent diversity in facial form, we remain exquisitely aware of even the most subtle craniofacial malformations. How is the molecular machinery so handily employed by Mother Nature to generate a normal craniofacial form? How does it react when perturbed by gene mutations and environmental teratogens? The objective of this chapter is to understand the process by which shape and form (i.e., morphogenesis) in the craniofacial region are controlled. Armed with this understanding, the phenotypic basis for a range of genetic mutations can be viewed in a new light. From this unique vantage point, we can also begin to contemplate strategies for the treatment and the future prevention of craniofacial birth defects.

I. IN THE BEGINNING...

For all intents and purposes, one can think of the process of craniofacial development as beginning as soon as the axes of the embryo are established. Over the past few decades, we have gained critical insights into these early stages of craniofacial development. A number of excellent reviews have been

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published recently that summarize these data, and interested readers are directed to these summaries (see Further Readings). This chapter will focus on later periods of craniofacial development (when the facial prominences begin to grow and fuse with one another) and on the times at which genetic disruptions and teratogenic exposures compromise normal craniofacial morphogenesis. These kinds of malformations are typically less severe than earlier patterning defects, but they also occur with greater frequency, and so they will be the focus of our chapter.

II. A LEXICON OF CRANIOFACIAL DEVELOPMENT

The craniofacial complex is comprised of the *chondrocranium,* which contributes to the bones of the base of the skull and encases the sense organs; the *splanchnocranium,* which gives rise to the pharyngeal region and contributes to the production of the jaws; and the *dermatocranium,* which consists of membrane bones that superficially invest the endoskeletal regions of the skull. Our attention is directed to the facial skeleton, which is derived from the chondrocranium (e.g., the sensory capsules of the nose and eyes) and the splanchnocranium (the jaws; Figure 30.1, A).

Craniofacial mesenchyme is derived from three mesodermal populations (the *prechordal plate,* the *lateral plate*, and the *paraxial mesoderm*) and the *cranial neural crest* (Noden, 1988). Vascular endothelial cells are derived from paraxial and lateral mesoderm, whereas pericytes enveloping these endothelial cells are derived from the cranial neural crest. Voluntary muscles of the head are derived largely from the prechordal and paraxial mesoderm, whereas the facial skeleton is derived from cranial neural crest and paraxial mesoderm (see Figure 30.1, B).

Bones in the craniofacial complex form through both *endochondral* and *intramembranous ossification.* In addition, other types of skeletal tissues, including *persistent* or *secondary cartilages*, are also found in the head (see Figure 30.1, C).

A common misperception is that cranial neural crest cells form bone exclusively through intramembranous ossification, whereas mesodermal cells form bone through endochondral ossification. Although the majority of cranial neural crest–derived bones form through intramembranous ossification, there are a number of neural crest–derived bones that undergo endochondral ossification, including the entire base of the skull and the bones in the second pharyngeal arch. Similarly, mesoderm-derived cells can undergo intramembranous ossification; this is readily apparent in the patella, the growth plate of the tibial tuberosity, regions of the ribs, and the clavicle. Therefore, the embryonic origin of a cell—whether neural crest or mesoderm—does not predict the mechanism by which it will form a skeletal element.

III. ORGANIZING THE CRANIOFACIAL PROMINENCES

The basic morphology of the face is established between the fourth and tenth weeks of human gestation by the development and subsequent fusion of the median nasal and lateral nasal prominences, which are subdivisions of the frontonasal prominence, and by the development of the maxillary and man-

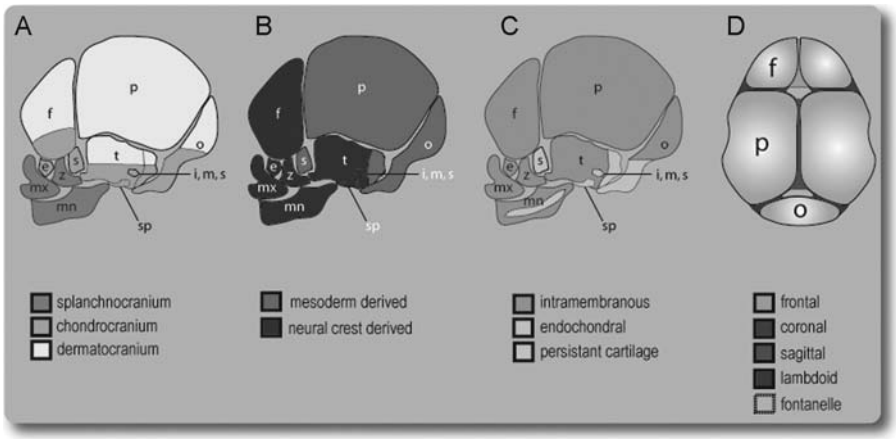

FIGURE 30.1 The classification of the cranial skeleton and sutures. The skull can be divided into many subcategories. **A,** Spatially, it is divided into the chondrocranium *(light green)*, which consists of the bones that make up the base of the skull and the skull vault; the splanchnocranium *(dark green)*, which consists of the bones of the lower face and jaw; and the dermatocranium *(yellow)*, which consists of the membrane bones that superficially invest the endoskeletal regions of the skull. **B,** On the basis of origin, the skull can be divided into two categories: bones derived from a neural crest lineage *(dark blue)* and bones derived from paraxial mesoderm *(pink)*. **C,** Bones of the skull can also be classified according to the mechanism by which they ossify. Some bones undergo endochondral ossification *(yellow)*, whereby the chondrocytes are invaded by the vasculature and ultimately replaced by bone. Other bones ossify via intramembranous ossification *(blue)*, during which chondrocytes differentiate directly into osteoblasts. In the skull, a third possibility exists: persistent or secondary cartilages *(green)* are skeletal elements that never ossify, instead retaining a cartilaginous character. **D,** The four major sutures of the skull. The frontal suture *(orange)* separates the frontal bones *(f)*. The coronal suture *(red)* separates the frontal bones from the parietal bones *(p)*. The sagittal suture *(blue)* separates the two parietal bones. The lambdoid suture separates the parietal bones from the occipital bones *(o)*. Fontanelles *(dotted black lines)* are large, soft, membrane-covered spaces that are interspersed between the calvaria and that eventually close with age. *Mx,* Maxilla; *mn,* mandible; *z,* zygomatic; *e,* ethmoid; *t,* temporal; *s,* sphenoid; *i,* incus; *m,* malleus; *s,* stapes; *sp,* styloid process of temporal bone. Bones not shown: nasal, lacrimal, vomer, and inferior nasal conchae. (See color insert.)

dibular prominences, which are derived from the first pharyngeal (or branchial) arch (Figure 30.2). These seven prominences expand, fuse, and then exhibit regions of localized growth that transform the relatively homogenous collection of prominences into facial features that are distinctive among different species of animals (Figure 30.3).

The prominences are largely composed of cranial neural crest cells surrounded on the outer surface by facial ectoderm and covered on the inner surfaces by neuroectoderm and pharyngeal endoderm. This tissue arrangement is achieved when neural crest cells migrate in well-defined streams from the dorsal neural tube into the future facial prominences (Figure 30.4). After this cellular influx, cranial neural crest cells proliferate, and the prominences expand until they are juxtaposed. Ultimately, the fusion of the median nasal, lateral nasal, maxillary, and mandibular prominences is the foundation for normal craniofacial development.

The middle and upper face are derived from the frontonasal prominence, whereas the sides of the face, the lower jaw, and the neck are derived from the first, second, third and fourth pharyngeal arches (the fifth arch fails to develop, and the sixth arch is a rudimentary structure; see Figure 30.4, A and B).

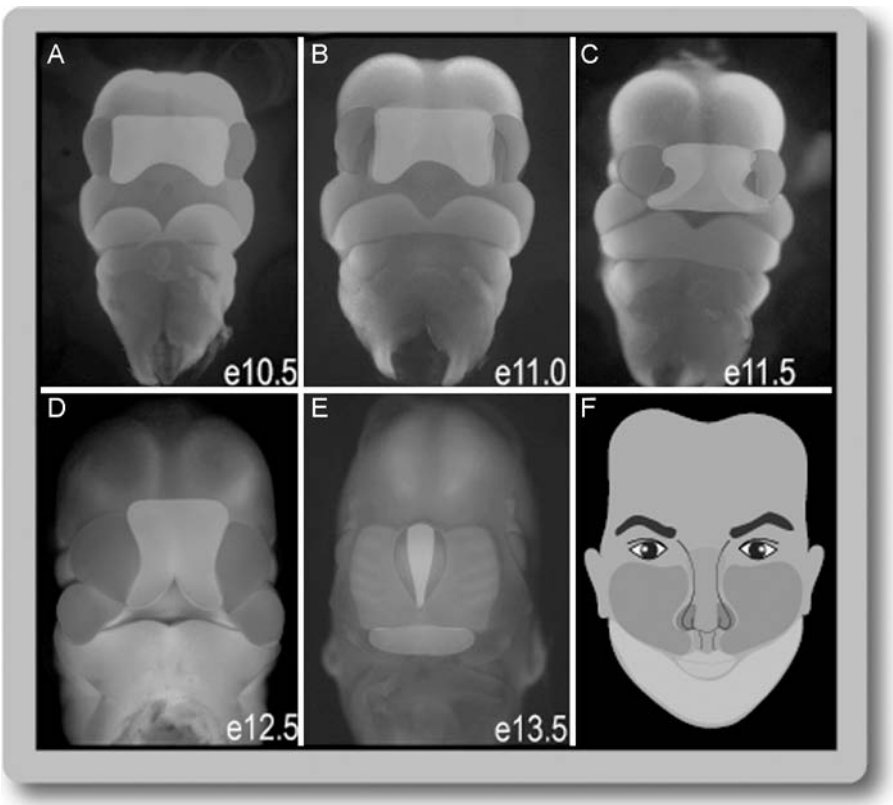

FIGURE 30.2 **A developmental series of murine embryos depicting the growth and fusion of the facial prominences. A,** At embryonic day 10.5, the seven facial prominences are evident. The first branchial arch has split into the maxillary *(red)* and mandibular *(yellow)* components. The lateral nasal *(green)* and medial nasal *(blue)* are present. **B** through **E,** As the embryo develops, the prominences grow, fuse, and are shaped into characteristic features of the vertebrate face by regionalized outgrowth. The mandibular prominences fuse with one another. The lateral nasal and the frontonasal fuse to form the nasal pit (future nostril). The maxillary prominences fuse with the lateral nasal and medial nasal to form a seamless continuum between the nose and the upper lip. **F,** Schematic representation of the contributions that the facial prominences make to the human face. (Drawings by Yvonne Y. Wang. See color insert.)

Each arch is composed of two mesenchymal and two epithelial tissues. The tissues are organized such that the cranial neural-crest–derived mesenchyme surrounds a core of mesoderm-derived mesenchyme (see Figure 30.4, C and D). Both mesenchymal populations are then encapsulated by a surface ectoderm and an internal pharyngeal endoderm (see Figure 30.4, C and D). In contrast, the upper face is composed of the neuroectoderm of the forebrain, a neural crest–derived mesenchyme, and a surface ectoderm (see Figure 30.4, E).

A. Patterning of the Pharyngeal Arches

In the neck and the lower jaw, the pharyngeal endoderm forms a series of pouches; this in effect creates regions where the pharyngeal endoderm and the surface ectoderm are juxtaposed, and these regions together form the pharyngeal clefts and the pharyngeal pouches (see Figure 30.4, D). The first pharyngeal pouch forms the primitive tympanic cavity and the auditory

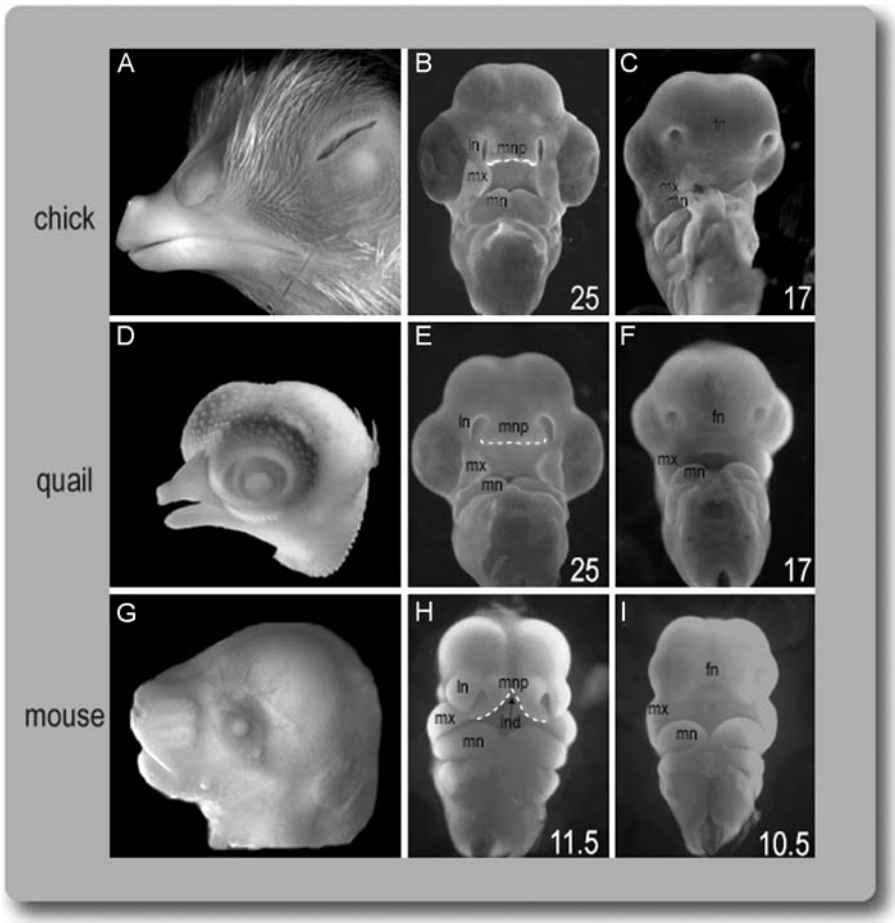

FIGURE 30.3 **The development of the facial prominences among different species. A,** Chick. **D,** Quail. **G,** Mouse. **C, F,** and **I,** The craniofacial complex of all vertebrates is initially very similar. During the early stages, it is hard to distinguish between avian and mammalian embryos, because the prominences are similar in shape and size. *mn,* Mandibular; *mx,* maxillary; *fn,* frontonasal. **B, E,** and **H,** Species variation becomes evident as regionalized outgrowths shape the prominences. The frontonasal *(fn)* has split into the lateral nasal prominence *(ln)* and the medial nasal prominence *(mnp)*. In avians, the regional growth of the medial nasal prominence is consistent across the prominence, thereby resulting in an even outward projection (**B** and **E**; *dotted line*) that predicts the upper beak. In murine embryos, regionalized outgrowth is restricted to the lateral aspect of the medial nasal prominence, thereby predicting the future infranasal depression *(ind)* (**H**; *dotted line*), a structure that is analogous to the human philtrum.

tube; the second pharyngeal pouch forms the palatine tonsil, and the third pharyngeal pouch forms the thymus, the parathyroid, and the thyroid glands.

The pharyngeal endoderm may appear to be a homogenous layer of epithelium; the nested patterns of gene expression, however, belie this uniformity. Genes in the bone morphogenetic protein (BMP), fibroblast growth factor (FGF), and Hedgehog families are expressed in highly dynamic and restricted patterns within the pharyngeal endoderm, and each plays crucial roles in the patterning and morphogenesis of structures derived from this epithelium (Graham and Smith, 2001).

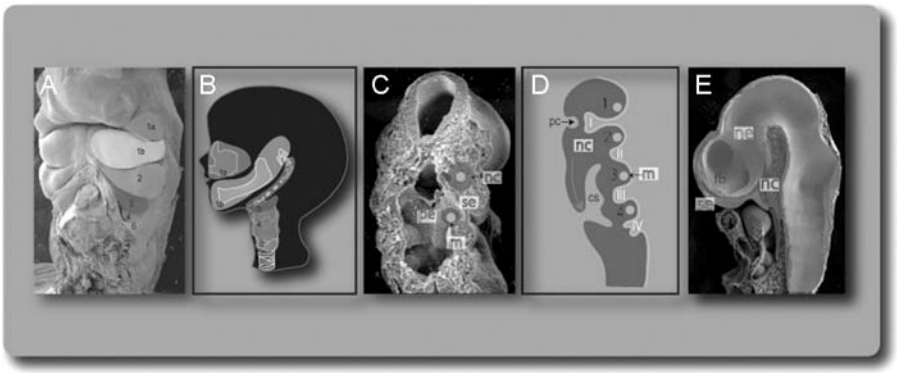

FIGURE 30.4 **The branchial arches. A,** Scanning electron image of the pharyngeal arches. The first four pharyngeal arches are indicated by different colors. The first arch has split into the maxillary *(1a, orange)* and mandibular *(1b, yellow)* components. The second arch *(2, green)*; the third arch *(3, purple)*; the forth arch *(4, pink)*, and the residual sixth arch *(6, blue)*. **B,** The skeletal derivatives of the pharyngeal arches. The arches and their corresponding derivatives are marked by matching colors. A detailed description of the derivatives of the pharyngeal arches is outlined in Table 30.1. **C,** The tissue composition of the pharyngeal arches. All of the pharyngeal arches are surrounded by an epithelial layer. The outer epithelial layer is derived from the surface ectoderm *(se, dotted blue line)*, whereas the inner (lumenal) epithelium originates from the pharyngeal endoderm *(pe, dotted green line)*. Neural crest cells *(nc, purple)* migrate into the epithelial-lined pouches and surround a core of mesoderm *(m, orange)*. **D,** The outgrowths of the pharyngeal clefts *(pc)* and pouches *(white Roman numerals)*. The second arch grows downward *(pink arrow)*, overlapping the second, third, and fourth pharyngeal clefts and forming the lateral cervical sinus of Hiss *(cs)*. As previously depicted, the arches *(1, 2, 3, 4)* are composed of a core of neural crest *(nc, purple)* and mesoderm *(m, orange)* that is surrounded by both surface ectoderm *(blue)* and pharyngeal endoderm *(green)*. **E,** Sagittal view illustrating the tissue components of the frontonasal prominence. The frontonasal prominence consists of an outer layer of surface ectoderm *(se, blue)* and an inner layer of neural crest *(nc, purple)*. The lumenal lining of the frontonasal prominence is the neural ectoderm *(ne, orange)* of the forebrain *(fb)* as opposed to the pharyngeal endoderm in the pharyngeal arches. (Drawings by Yvonne Y. Wang. See color insert.)

B. Patterning of the Surface Ectoderm

Pharyngeal endoderm is not the only epithelium in the face that contains patterning information: the ectoderm covering the facial prominences is also a source of organizing signals that direct the underlying neural crest mesenchyme to proliferate and differentiate into the skeletal tissues of the face. Cranial neural crest cells destined for the frontonasal prominence migrate over the forebrain. After they take up residence in the prominence, they are sandwiched between the neural ectoderm of the forebrain and the facial ectoderm. Both epithelia provide instructive cues that direct patterning within this population of mesenchyme. They achieve this effect via organizing centers, which are defined regions of epithelia that have the ability to instruct and program the fate of cells in their vicinity.

The invagination of the pharyngeal surface ectoderm forms the pharyngeal clefts (see Figure 30.4, D). The first pharyngeal cleft forms the external auditory meatus, and the second pharyngeal cleft forms the cervical sinus. In normal development, the cervical sinus narrows to form a small channel, which is referred to as the *cervical duct;* this will eventually be obliterated. When the cervical sinus is retained, the consequence is the formation of branchial cysts and eventually a branchial sinus or a branchial fistula that connects the skin to the lumen of the foregut. The third and more posterior pharyngeal

clefts are encased in the ventral outgrowth of the second pharyngeal cleft (see Figure 30.4, D). The derivatives of the arches are summarized in Table 30.1.

Some of the same molecules expressed in the pharyngeal endoderm are also expressed in the facial ectoderm: Sonic hedgehog (Shh), members of the FGF and BMP families, and a number of Wnt genes show localized, ever-changing patterns of expression in this surface epithelium. Their functions in this tissue layer are critical for the proper development of the middle and upper face, and a host of craniofacial malformations are associated with disruptions in their function within this tissue (reviewed in Helms et al., 2005).

C. Patterning in the Forebrain Neuroectoderm

The forebrain neuroectoderm also participates in craniofacial patterning, but it does so in ways that are not clearly defined. For example, the forebrain acts as a structural support for facial development, and this is exemplified by the clinical condition holoprosencephaly (HPE). Although the presentation of HPE is heterogeneous (described later), there are common features that indicate that the impaired midline cleavage of the forebrain typically results in the loss of facial elements derived from the midline frontonasal prominence. Likewise, in conditions in which the synthesis, secretion, or activity of neuroectodermal growth factors (e.g., retinoids, FGFs) is disrupted (described later), the resulting craniofacial defects may be attributable to a collapse of the forebrain scaffold as a result of apoptosis in the neuroectoderm.

These types of severe brain anomalies are often accompanied by facial malformations, and, until recently, the extent of the facial malformation was considered a direct reflection of the underlying forebrain anomaly. This association between brain and facial defects in HPE led to the premise that "the face predicts the brain." This concept provided physicians with a framework for the diagnosis and prognosis of HPE and other conditions for more than 40 years. However, with the advent of sophisticated brain imaging techniques (e.g., magnetic resonance imaging) and advanced three- and four-dimensional fetal sonographic imaging coupled with mutation analyses, the link between brain and facial malformations has become less clear. We now

TABLE 30.1 Derivatives of the Pharangeal Arches

Arch	Skeletal Element	Musculature	Nerve
First	Incus, malleus, zygomatic, squamous, part of the temporal, mandible, maxilla	Muscles of mastication	Trigeminal (V)
Second	Stapes, styloid process of temporal bone, stylohyoid ligament, lesser horn and body of hyoid bone	Muscles of facial expression	Facial (VII)
Third	Greater horns and lower body of the hyoid (throat)	Muscles of the stylopharyngeus	Glossopharyngeal (IX)
Fourth and sixth	Cartilages of the larynx	Muscles of pharynx constriction, muscles of phonation, palatoglossus (tongue), muscles of the upper esophagus	Vagus (X)

know that patients with alobar HPE can exhibit normal facial appearances. Conversely, patients with an HPE gene mutation and facial features suggesting the most severe form of HPE can show normal brain anatomy on magnetic resonance imaging. Consequently, the traditional classification scheme for HPE and the "face predicts the brain" maxim are sometimes inconsistent with molecular genetic data and advanced imaging techniques.

IV. ASSEMBLING THE PIECES OF THE CRANIOFACIAL COMPLEX

A. The Median and Lateral Nasal Prominences

The frontonasal prominence contributes to midline features, including the forehead, the middle of the nose, the philtrum of the upper lip, and the primary palate. The frontonasal prominence has two components: the median nasal prominence and the lateral nasal prominences. Unlike the other facial prominences, the growth and morphogenesis of the frontonasal prominence are influenced to a large degree by molecular instructions emanating from the forebrain neuroectoderm. When molecular signals from the forebrain are disrupted, the consequence is invariably a disruption in the patterning and growth within the frontonasal prominence. One of the best studied of these malformations is the condition of HPE (described later). The other component of the frontonasal prominence is the lateral nasal prominences, which contribute to the sides (ala) of the nose. As with the frontonasal prominence, the lateral nasal prominence is composed of surface ectoderm, neural crest–derived mesenchyme, and an underlying layer of neuroectoderm (forebrain). The tissues and molecular signals that regulate the morphogenesis of the lateral nasal prominences are poorly understood, but they presumably involve the surface ectoderm and perhaps the forebrain neuroectoderm as well.

B. Maxillary and Mandibular Prominences

The maxillary and mandibular prominences are derived from the first arch. Unlike the frontonasal prominence, the first arch does not contain a neuroectodermal component. Instead, the first arch has an underlying layer of pharyngeal endoderm. The maxillary prominences give rise to the upper lip and jaw and the secondary palate. Any aberrant growth or fusion between these prominences can result in cleft lip and/or palate (described later). The mandibular prominence is also derived from the first arch. The major derivatives of the mandibular prominence are the lower lip and jaw (see Figure 30.4).

Although current dogma states that the ventral region of the first pharyngeal arch gives rise to the mandible and that the dorsal region gives rise to the maxilla, recent fate-mapping studies have indicated that the ventral portion of the first arch actually gives rise to both maxillary and mandibular skeletal elements (Cerny et al., 2004; Lee et al., 2004). Further, it has been proposed that the maxillary prominence and its skeletal derivatives are not derived from the first pharyngeal arch but rather from a separate maxillary condensation that occurs between the eye and the maxillomandibular cleft (Lee et al., 2004). Although the actual origin of these structures is still up for debate, etiologic studies such as these are essential to understanding both the normal and abnormal development of facial prominences.

C. The Cranial Skull Vault

The cranial vault encases and protects the brain. The ability of the brain to grow and of the skull to accommodate this growth is permitted by fibrous membranes that exist between the flat bones of the skull, which are called *sutures*. Because the bones of the cranial vault do not complete their growth during fetal life, sutures permit growth at the edges of the bone. There are four major sutures: the *frontal suture* separates the frontal bones; the *coronal suture* separates the frontal and parietal bones; the *sagittal suture* is between the parietal bones; and the *lambdoid suture* separates the parietal bones from the occipital bone (see Figure 30.1, D). At the intersection between sutures, interspersed between the calvaria, are large, soft, membrane-covered spaces or *fontanelles* (see Figure 30.1, D). Fontanelles occupy the areas between the corners of the calvaria at birth. Although it has been suggested that fontanelles and sutures permit the skull vault to deform as it passes through the birth canal, this hypothesis is erroneous, because animals that do not pass through a birth canal (amniotes, including birds and their reptilian ancestors) also have sutures.

Shortly after mesenchymal cells aggregate and form the skeletogenic condensations of the calvaria, there is another decision the cells must make: whether to differentiate into chondrocytes that progress from an antiangiogenic status to an angiogenic phase in which they are invaded by the vasculature and ultimately replaced by bone (endochondral ossification) or to differentiate directly into osteoblasts (intramembranous ossification). In the head, there is a third possibility: some mesenchymal cells differentiate into a type of chondrocyte that never undergoes an angiogenic switch and thus is retained instead of being replaced by the vasculature. This latter type of cartilage is referred to as *persistent cartilage* (e.g., Meckel's cartilage; see Figure 30.1, C).

Intramembranous ossification is not unique to the craniofacial complex, although it is more prevalent in the head than in other regions of the body. This has led to a common misconception: that cranial neural crest cells form bone exclusively through intramembranous ossification and that mesoderm-derived skeletal elements form bone exclusively through endochondral ossification. In actuality, cranial neural crest cells and mesodermal cells are equally capable of generating skeletal tissues through intramembranous or endochondral ossification (see Figure 30.1, C).

V. DIFFERENTIATING THE CRANIOFACIAL COMPLEX

During embryonic development, the facial prominences among the species look remarkably similar. How and when do the faces of various animals gain their unique identities? Recent studies suggest that a combinatorial code of gene expression and tissue interactions control differential growth in the individual prominences. Dogs are the ideal example of this concept; think of bulldogs and dachshunds, and one immediately appreciates the variation in the differential growth of the frontonasal prominence. Differential growth is also what allows the frontonasal prominence of a mouse to form its characteristic infranasal depression and the frontonasal prominence of the chick to form the upper beak. If the differential growth of the prominences accounts for the vastly different facial structures among species, then what molecular cues

are involved, and on which tissues are they acting? Several studies have tried to address this question using a multitude of inventive experiments.

A. Transforming a Mandible into a Maxilla

All pharyngeal arches are comprised of similar tissues; to investigate the mechanisms that specify the identity of skeletal elements within these arches, investigators inactivated two members of the *distalless* family of transcription factors, Dlx5 and Dlx6 (Depew et al., 2002). These homeobox genes are expressed in the mandibular prominence but not in the maxillary prominence. By knocking out both genes, the mandible gained a maxillary-like expression pattern, and the phenotypic result was that the lower jaws transformed into upper jaws. Thus, the mutant animal had duplicated sets of whisker primordia and upper jaw skeletal elements. These data indicate that the facial prominences are interchangeable to a remarkable degree and that the genes expressed within a prominence specify its identity within the craniofacial complex.

B. Transforming a Maxilla into a Frontonasal Prominence

Additional experiments have been undertaken to define the molecules responsible for imparting identity on the frontonasal prominence and the maxilla. BMPs and the vitamin-A–derived retinoic acid are two molecules that are implicated in the patterning of the middle and upper face. In these experiments, beads soaked in the BMP antagonist Noggin and retinoic acid were implanted into the maxillary prominence of a chicken embryo. The phenotypic result was the transformation of the maxillary prominence into a frontonasal prominence. These results further argue the malleability of the prominences, and they suggest that two of the molecules that confer an identity onto the cells of the maxilla include BMPs and retinoic acid (Lee et al., 2001).

C. Neural Crest Cells Contain Some Species-Specific Patterning Information

Studies have also been performed to determine which tissues contain the patterning information that regulates species-specific craniofacial form. The neural crest was an obvious first candidate, because this cell population contributes to the majority of the bones and connective tissues within the face. Investigators wondered whether neural crest cells contain the information responsible for the differential growth of the frontonasal prominence in different species, and they addressed this question by grafting neural crest cells bound for the frontonasal prominence between two avian species. Thus, duck neural crest cells were transplanted into quail embryos ("duails"), and quail neural crest cells were transplanted into duck embryos ("qucks"). The resulting chimeras were examined for any phenotypic variations, and what the researchers found was striking: the qucks had the beak of a quail on the body of a duck, whereas the duails had the bill of a duck on the body of a quail. These results suggest that species-specific craniofacial morphology is determined in large part by molecular cues emanating from the neural crest (Schneider and Helms, 2003).

D. Modifying Bone Morphogenetic Protein Signaling Alters Facial Morphology

From the finches of Galapagos to the cichlids of Africa to the honeycreepers of Hawaii, we have been shown how ecologic niches can dictate facial form.

Although this extraordinary variation in beak or jaw morphology is associated with the use of a variety of environments, its molecular basis remains unknown. Using the comparative analysis of expression patterns of various growth factors, investigators have begun to address this question in various avian species. BMP4 has emerged as a signal that may play a role in dictating facial morphology. In avians, localized zones of BMP4 expression appear to demarcate regions of differential growth. When BMP4 was misexpressed, the beaks of the treated embryos underwent a morphologic transformation that made them appear much broader and deeper (Abzhanov et al., 2004). A similar type of morphologic transformation has taken place through the evolution of the Galapagos finch, and there are some data that suggest that the mechanisms by which Mother Nature shaped the finch beak are analogous to what scientists achieved in the laboratory. These results in birds suggest that BMP signaling plays a role in the modulation of beak size and shape.

The concept of transforming a beak to a bill is an intriguing one. However, can we step outside of the confines of one species and take this concept a step further? If altering the expression of BMP4 can change the shape of a beak, can altering the expression of different molecules transform a bird's beak into a mouse's muzzle? Hasn't evolution shown us this is possible? Our best bet is taking cues from the transformations that nature has already produced.

VI. CONGENITAL DISORDERS

Each component of the face—the forehead, the nose, the cheeks, the lips, the jaws, and the chin—arises from a highly coordinated series of morphogenetic events, including extensive cell migrations and extracellular matrix remodeling, the proliferation and differentiation of cells into skeletal and connective tissues, and the assembly of musculature. Perturbations during any of these developmental steps can prevent subsequent fusion among the individual parts that are required to produce a whole face with structural integrity and functional unity.

A. Facial Clefting

The failure of parts of the face to join together is called *clefting*. The vast majority of clefts are found along lines of fusion, and they can range from severe (clefts affecting the nose, eyes, and brain) to mild (clefts involving the vermillion border of the lip or that are limited to the hard tissue). Clefting malformations occur in approximately 1 in every 700 births, thus making them one of the most prevalent craniofacial birth defects. Clefts are the manifestation of defects in the rate, the timing, or the extent of the outgrowth of the facial prominences. Therefore, understanding the causes of these clefts begins with an understanding of how the prominences normally grow out and fuse.

1. Secondary Palatal Clefting

In mammals, the secondary palate separates the nasal passage from the pharynx; it forms from the outgrowth of the medial domain of the maxillary prominences (Figure 30.5, A). In mammals, the maxillary prominences begin

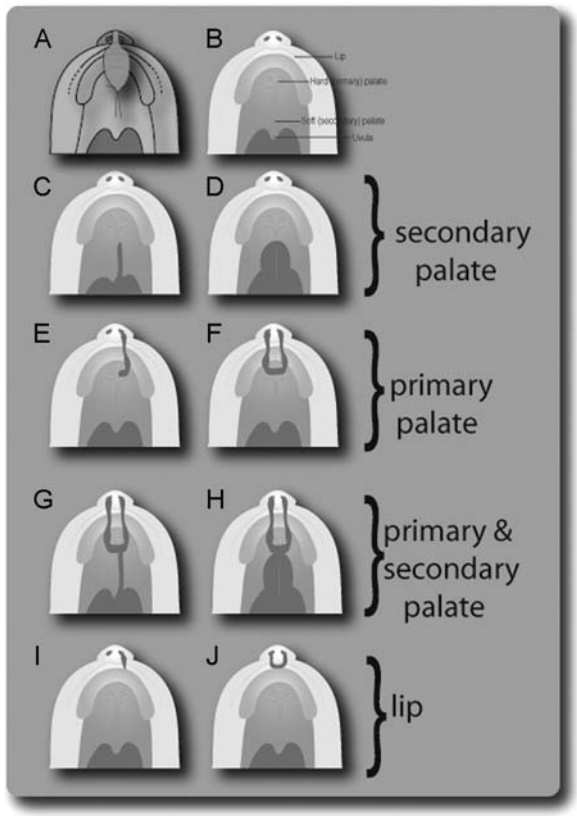

FIGURE 30.5 **Schematic diagram illustrating various forms of clefting. A,** A schematic diagram of a ventral view of the roof of the mouth. The primary (or hard) palate is derived from the frontonasal prominence, whereas the secondary (or soft) palate is derived from the maxillary portion of the first pharyngeal arch. The alae of the nose are derived from lateral nasal prominences. **B,** A ventral view of an unaffected (nonclefted) palate. **C,** Unilateral clefting of the secondary palate. **D,** Bilateral clefting of the secondary palate. **E,** Unilateral clefting of the primary palate. **F,** Bilateral clefting of the primary palate. **G,** Bilateral clefting of the primary palate coupled with unilateral clefting of the secondary palate. **H,** Bilateral clefting of the primary palate coupled with bilateral clefting of the secondary palate. **I,** Unilateral cleft lip. **J,** Bilateral cleft lip. (Drawings by Yvonne Y. Wang.)

as small buds off of the first pharyngeal arch that initially grow outward and that then grow caudally until they flank the embryonic tongue. The secondary palate arises from condensations of neural crest mesenchyme within the maxillary prominences. The tongue is initially in an elevated (dorsal) position, but, as the mandibular prominences grow outward, they displace the tongue with them, and it assumes its more ventral position. The medial regions of the maxillary prominences simultaneously begin to elevate, like swinging doors, to a more dorsal position. This movement brings the palatal shelves into proximity with one another so that the epithelia covering the prominences are in contact. This region of the palatal shelf epithelium is referred to as the *medial edge epithelia*, and the epithelial cells in this region begin to coalesce to form the *medial epithelial seam* (MES). Epithelial cells comprising the MES are then removed, most likely through their selective programmed cell death, and the mesenchyme of the palatal shelves becomes contiguous. If one

appreciates the fact that all of these morphologic movements must occur in synchrony, then it is easy to understand how disruptions at any stage of growth or cell movement can perturb the process of fusion, with the untoward consequence being a cleft (Figure 30.5, C, D, E, and F). For example, if the mandibular prominences fail to grow outward, the tongue will not drop from its dorsal position to a more ventral location; as a consequence, the tongue remains interposed between the maxillary prominences, and the result is a cleft of the secondary palate caused by the inadequate development of the lower jaw. Insufficient outgrowth of the maxillary prominences as a result of inadequate neural crest cell proliferation can lead to the failure of the palatal shelves to approximate; in these cases, the secondary palatal cleft is attributed to palatal insufficiency. Secondary palatal clefts can also form because of the retention of the MES between the fusing prominences; in these cases, the overlying soft tissues may fuse, but the palatal bones do not, and the result is an incomplete palatal cleft. Some variations of clefts within the secondary palate are depicted in Figure 30.5.

The genetic/molecular basis of cleft palate is as multifarious as its clinical presentation: disruptions of the patterning, migration, proliferation, and differentiation of cells or of the remodeling of the extracellular matrix can result in clefting. These perturbations can be caused by a host of genetic, mechanical, and teratogenic factors. Insights into the etiopathogenesis of clefting have predominantly come from studies in mammals and birds. Mammals are among the few animals in which the secondary palate is fused, whereas the secondary palate of a bird remains naturally cleft throughout the life of the animal. Thus, comparing the molecular/cellular processes that regulate the patterned outgrowth and fusion of the palatal shelves in birds and mice provides a view into the mechanisms that direct the outgrowth of facial prominences.

Transforming growth factor beta 3 (TGFβ3) is a member of the TGFβ superfamily of proteins, of which three mammalian isoforms have been identified. These molecules have been implicated in cell proliferation, differentiation, migration, the regulation of extracellular matrix deposition, and epithelial–mesenchymal transformation. Relevant to its potential role in facial clefting is the fact that TGFβ3 is expressed by medial edge epithelia cells just before the fusion of palatal shelves and that it ceases to be expressed shortly after the MES forms. If TGFβ3 signaling is blocked, then palatal shelves fail to fuse. Mice that are homozygous for a null mutation in TGFβ3 exhibit isolated cleft secondary palate. In these newborn mice, the palatal shelves approximate and adhere, but the MES remains, and mesenchymal confluence does not occur. These and other studies indicate that TGFβ3 is involved in mediating the removal of the MES, and this leads to the confluence of the palatal mesenchyme. However, precisely how the MES is removed remains unclear. There are at least three possible explanations for the disappearance of the seam. The first possibility is that the midline epithelial cells undergo programmed cell death, thus allowing mesenchymal cells to move into the space previously occupied by the epithelial cells. A second possibility is that the epithelial cells migrate from a midline position and become contiguous with the adjacent oral or nasal epithelium. A third possibility is that the midline epithelial cells are transformed into mesenchymal cells. In reality, a combination of all three mechanisms may be operating, and their effects may, to varying degrees, depend on which prominence is undergoing fusion.

2. Primary Palatal Clefting and Clefting of the Lip

In all vertebrates, the frontonasal prominence gives rise to the primary palate, the midline of the upper lip, and the infranasal depression (philtrum) (see Figure 30.5, A). In mammals, the primary palate contains the upper incisors. The frontonasal prominence in the mouse develops as two lobes separated by a fissure, again predicting the shape of the future lip. In birds, the frontonasal prominence begins to elongate, thus predicting the shape of the future beak (see Figure 30.3). A failure in the growth of the median and lateral nasal primordia precludes the subsequent fusion of these structures. As a consequence, clefts develop between their derivatives. In the mildest cases, the clefts may be limited to the vermillion border of the lip. In progressively more severe cases, the cleft develops through the tissues of the lip (unilateral or bilateral cleft lip), and they can also involve the side of the nose (typically referred to as *oblique clefts;* see Figure 30.5, I and J).

Defects in Shh have been linked to cleft lip. Shh expression is limited to the ectoderm of the frontonasal and maxillary prominence, which give rise to the upper beak, the primary palate, the sides of the face, and the secondary palate. Mice carrying null mutations in Shh exhibit HPE, a condition that, in its most severe form, is manifested as cyclopia. Whether Shh plays a role in later steps of craniofacial morphogenesis cannot be answered via the Shh mouse, because the animal shows an almost complete lack of cranial structures as a result of early patterning defects in the neural plate and the neural tube.

To clarify the contribution of Shh signaling to craniofacial patterning, small regions of Shh-positive ectoderm were excised from the frontonasal processes of stage 25 chick embryos, with are taken to leave the underlying mesenchyme intact. The ablation of this region resulted in the inhibition of the ensuing growth of the frontonasal process and a subsequent failure of the frontonasal process to fuse with the other primordia. This creates a defect in chick embryos that is analogous to the bilateral cleft lip and palate seen in humans.

B. Teratogen-Induced Facial Defects

Teratogens are chemicals, drugs, or infectious agents that, when introduced into the embryonic environment, have the potential to produce birth defects, including facial clefting. By studying the molecular and cellular bases by which teratogens exert their effects, we can gain a better understanding of the molecular and cellular regulation of normal development. Although the abnormalities that arise after exposure to a specific teratogen may be highly variable, they are usually reproducible, because teratogens have distinct mechanisms of action, and they are selective with regard to their target cells, tissues, and organs.

1. What Teratogens Reveal about Craniofacial Development

Four general factors may account for the range of phenotypic effects that can arise after exposure to a teratogenic agent: (1) differences in the concentration or method of teratogen delivery; (2) the timing of exposure during embryonic development; (3) variations in the susceptibility of individuals as a result of diverse genetic backgrounds; and (4) synergistic interactions among various compounds. All of these factors can lead to a continuum in the severity of birth defects in one species. Numerous substances have been identified that have teratogenic effects during craniofacial development. In particular,

we will focus on two teratogens—retinoic acid and alcohol—that disrupt the formation of the facial prominences and that can lead to clefting.

2. Retinoic Acid and Alcohol

Clinical and experimental data generated during the past 70 years clearly demonstrate that retinoic acid, which is a metabolite of vitamin A, can act as a powerful teratogen during embryogenesis. Both excesses and deficiencies of retinoic acid can lead to severe abnormalities in a variety of tissues. The brain and the face in particular appear to be especially sensitive to changes in the availability of retinoic acid during development. Nervous system defects (e.g., microphthalmia, HPE) and facial anomalies (e.g., midfacial hypoplasia, cleft lip/palate) are all potential adverse outcomes that may occur after exposure to retinoic acid.

The severity and extent of the craniofacial defects appear to relate to the developmental stage at which the exposure occurs, the dosage of the retinoic acid, and the particular tissue that is subjected to the exposure. When it is delivered to the developing facial prominences, retinoic acid produces a wide range of morphologic defects. Treatment with high doses of all-trans retinoic acid gives rise to embryos in which the frontonasal mass is entirely absent; however, at this same dosage and embryologic age, the mandibular process remains unaffected. Two questions arise from these types of results. First, what accounts for the loss of the frontonasal prominence? Second, why is the mandibular prominence unaffected by treatment with the same dose of retinoic acid? Most evidence suggests that retinoic acid exerts its teratogenic effects by disrupting the expression of Shh (Helms et al., 1994; 1997). For example, a deficiency in retinoic acid results in the loss of Shh signaling; the phenotypic effect creates a holoprosencephalic phenotype (described later). Alternatively, excesses in retinoic acid ectopically induce Shh, and the resulting phenotypes include facial clefting as a result of hypertelorism (an increased distance between the eyes) and, in extreme cases, duplications of facial structures.

Maternal alcohol consumption in humans causes a wide range of birth defects, and a subset of these defects has been ascribed to disruptions in endogenous retinoid biosynthesis or in the metabolism of retinoic acid. In the craniofacial complex, one function of retinoid signaling is the regulation of Shh (Helms et al., 1994). For example, when endogenous retinoid signaling is inhibited during embryogenesis, Shh expression in the craniofacial tissues is blocked; consequently, programmed cell death is increased. The net effect is a fused telencephalon, cyclopia or severe hypotelorism, and facial clefting. In zebrafish, exogenous retinoic acid initially leads to a decrease in or an inhibition of Shh expression, but this later causes ectopic Shh expression, just as it does in avians. The molecular basis for this repression/induction is still not clear. Nonetheless, studies carried out in multiple animal models demonstrate that one mechanism by which alcohol exerts its teratogenic effects is through the misregulation of retinoid signaling, which in turn disrupts the Shh signaling pathway.

C. Shh and Holoprosencephaly

HPE is a heterogeneous disorder that is characterized by a variable penetrance, multiple causes, and an astonishingly broad phenotypic profile. In its

most severe form, HPE manifests as cyclopia (from the Greek *kyklops,* meaning "circle or wheel"). The cyclopic HPE phenotype is incompatible with life, and this is attested to by the high spontaneous abortion rate of HPE fetuses (1 in 250). The few HPE fetuses that survive to birth (1 in 10,000 to 20,000 live births) exhibit craniofacial defects ranging from severe brain anomalies with midline clefting to relatively mild cleft lip and palate. Microforms of HPE also exist in which the clinical manifestation of the syndrome is as subtle as a single central incisor tooth or close-set eyes. Some individuals with HPE can also have completely normal facial appearances. Hence, its description as a "malformation sequence" is a most accurate moniker: HPE is a perplexingly broad spectrum of anomalies that lacks any constant anatomic feature.

Although the vast majority of HPE cases are considered sporadic, familial cases of HPE have been documented. In 1996, mutations in the murine and human Shh genes were the first to be causally linked to HPE. Since then, a number of other genes in the Shh pathway have been associated with HPE, including the Hedgehog receptor Patched (Ptch) and one Hedgehog target gene encoded by the transcription factor Gli2. Two other transcription factors, Zic2 and Six3, and the homeodomain protein 5′-TG-3′-interacting factor are also linked to human HPE.

These HPE mutations fall into the four major classes of genetic disruptions: (1) chromosomal abnormalities involving aneuploidy; (2) translocations; (3) deletions; and (4) missense and nonsense mutations. As of the writing of this chapter, Shh remains the most commonly identified gene in HPE, and it is mutated in both familial and sporadic cases.

Ten years have passed since the first genetic mutation of the human Shh gene was identified in human HPE; during the intervening decade, mutation analyses have indicated that HPE is a multigenic disorder. Although considerable progress has been made, our understanding of how particular gene disruptions actually cause HPE malformations remains nebulous. Uncovering the basis for the HPE represents a unique challenge, and the key to unlocking this disease will undoubtedly rest in the coordinated efforts of geneticists, clinicians, and developmental biologists.

1. Genetic Mutations Causing Holoprosencephaly

Although the vast majority of HPE cases have no known cause, some genetic progress has been made. Many genes with in the Hedgehog pathway have been implicated in this disease. For example, mutations in Ptch, which lead to truncations of the C-terminus, render the protein unable to repress Smoothened activity, and the net result is the constitutive activation of the Shh pathway. Basal cell nevus syndrome (also known as *Gorlin syndrome*) is an autosomal-dominant disorder caused by such a gain-of-function mutation in Ptch1. The most serious complication of this disorder is the predisposition to basal cell carcinomas and medulloblastomas. These patients may also exhibit dysmorphic facial features that consist of strabismus, cleft palate, and keratocysts of the jaw.

D. Craniosynostoses

Defects in proper skull development can result in multiple anomalies, the most common of which are a heterogeneous class of dysmorphologies known as the *craniosynostoses*. In general, these conditions are characterized by the premature fusion of a suture that, in the presence of continued brain growth, results

in gross malformations of the head. The craniosynostoses are categorized by virtue of the suture involved and by the presence of additional anomalies involving the organs, including the limbs, digits, and the central nervous system. In recent years, gene mutations associated with many of these craniosynostotic syndromes have been identified (described later). What remains to be determined is how these particular gene disruptions lead to disruptions in cell behavior and ultimately produce the craniofacial phenotypes associated with each disorder.

Craniosynostosis is defined as the premature fusion of any cranial suture. It is a fairly common (1 in every 2500 live births) developmental disorder (Hunter and Rudd, 1976; 1977; Lajeunie et al., 1995) that is characterized by premature suture fusion, which prevents normal cranial vault enlargement and results in numerous morphologic and physiologic abnormalities, including dysmorphic cranial shape, hydrocephalus (abnormal accumulation of cerebrospinal fluid in the ventricles of the brain), elevated intracranial pressure, deafness, blindness, mid-face hypoplasia, and compromised airways (Posnick, 2000). Premature suture fusion can occur either as an isolated condition or as part of several syndromes involving a myriad of abnormalities (Wilkie and Morriss-Kay, 2001). Numerous syndromes exist as a result of the improper fusion of the cranial sutures.

1. Associated Syndromes and Their Molecular Basis

A variety of syndromes characterized by craniosynostosis are the result of dominant mutations in genes encoding FGF receptors (FGFR1 and FGFR2), and this results in a constitutive activation of the receptor (gain of function). FGF signaling pathways are involved in the regulation of a variety of developmental processes, including endochondral and intramembranous bone formation (Ornitz and Marie, 2002). Below we examine some syndromes associated with craniosynostoses and try to accurately describe the published data that link them with a genetic component.

2. Phenotypic Presentation of Crouzon and Apert Syndromes

Crouzon (1 in every 25,000 to 65,000 live births) and Apert (1 in every 65,000 to 150,000 live births) syndromes are common craniosynostotic syndromes, and, together, they account for approximately 9% of all reported cases of craniosynostosis (Mooney and Siegel, 2002). Patients with Crouzon syndrome present with shallow ocular orbits, proptosis (bulging of the eyes), hypertelorism (an abnormally increased distance between the eyes), and maxillary hypoplasia (the incomplete or arrested development of upper jaw). Apert patients present with acrocephaly (the top of the skull assumes a cone shape), wide-open fontanelles at birth, hypertelorism, shallow ocular orbits, maxillary hypoplasia, and cleft palate. Defining differences between the Apert and Crouzon syndromes are the limb deformities that accompany Apert syndrome (Mooney and Siegel, 2002). Patients with Apert syndrome often have hand and foot syndactyly (the fusion of the digits; Mooney and Siegel, 2002; Wilkie et al., 1995).

3. Phenotypic Presentation of Pfeiffer Syndrome

Affecting approximately 1 in every 100,000 individuals, Pfeiffer syndrome involves craniosynostosis; bulging, wide-set eyes; an underdeveloped upper

jaw; and a beaked nose. In approximately half of cases, hearing loss and dental problems are additionally noted. Pfeiffer syndrome can be categorized into three subtypes. Type 1 or "classic" is described above, and types 2 and 3 are more severe forms that typically involve the classic symptoms compounded by problems with the nervous system. Type 2 is distinguished from type 3 by the more extensive fusion of bones in the skull, which leads to a "cloverleaf"-shaped head. Pfeiffer syndrome has been linked to mutations in both FGFR1 and FGFR2. Typically, the FGFR1 mutation is a missense mutation within exon IIIa, which affects the extracellular tyrosine kinase linker domain.

4. Activating Mutations in Fibroblast Growth Factor Receptors Causes Craniosynostoses

FGFRs have been linked to craniosynostosis (Malcolm and Reardon, 1996; Mooney and Siegel, 2002). Mutations in FGFR2 in particular have been linked to both Crouzon (Reardon et al., 1994; Gorry et al., 1995; Hollway et al., 1997; Li et al., 1995; Passos-Bueno et al., 1999) and Apert (Anderson et al., 1998b; Hollway et al., 1997; Park et al., 1995; Wilkie et al., 1995) syndromes. The FGFR2 mutation in Apert syndrome causes a change in the dissociation kinetics between FGF2 and FGFR2. As a result, the ligand stays bound to the receptor for a longer period of time, and this results in a constitutive activation (Anderson et al., 1998a). A similar mechanism is hypothesized to produce the defects seen in Crouzon syndrome as well (Mooney and Siegel, 2002). Notwithstanding, the majority of Apert and Crouzon cases are sporadic, which signifies that random mutations or an environmental insult to the genetic code are most likely responsible (Wilkie, 1997). Even with all of the genetic progress made toward understanding craniosynostosis and related syndromes, a remaining conundrum of cause and effect exists. The mechanism involving exactly how these gene defects manifest themselves in a craniosynostotic phenotype is yet to be uncovered.

5. The Saethre–Chotzen Syndrome and Twist Mutations

Saethre–Chotzen syndrome occurs in 1 in every 25,000 to 50,000 live births. The symptoms of this syndrome include turriplagiocephaly (the asymmetrical distortion of the skull), facial asymmetry, low hairline, proptosis, a characteristic slanting of palpebral fissures, nasal deviation with a high bridge, angled ears, torticollis (a wry neck), and neurosensorial hypoacousia (a partial hearing loss). However, Saethre–Chotzen syndrome exhibits a unique genetic linkage. Mutations in Twist, which is a basic helix–loop–helix transcription factor (Murray et al., 1992) have been genetically linked to this syndrome.

Twist was originally noted as a candidate gene for this condition because its expression pattern and mutant phenotypes in the mouse are consistent with the Saethre–Chotzen phenotype (Howard et al., 1997). Genetic mapping and mutational analysis revealed that the area occupied by Twist on human chromosome 7p21-p22 contained nonsense, missense, insertion, and deletion mutations in patients with Saethre–Chotzen syndrome (Howard et al., 1997). Typically, mutations in the gene resulting in Saethre–Chotzen syndrome affect the DNA-binding, helix-I, and loop domains, or they result in the premature termination of the protein. Studies in Drosophila indicate that Twist may affect the transcription of FGFRs. The current hypothesis is that Twist may function as an upstream regulator of FGFRs (Howard et al., 1997).

6. Boston-type Craniosynostosis and Mutations in Msx2

Boston-type craniosynostosis is yet another autosomal-dominant disorder that results in the premature fusion of calvaria and subsequent abnormalities in skull shape (Liu et al., 1995; Ma et al., 1996). Patients with this condition bear a mutated copy of the homeodomain protein Msx2 (Jabs et al., 1993; Ma et al., 1996). Msx2 is a transcription factor that is expressed in the neural-crest–derived mesenchyme of the pharyngeal arches and at birth in the osteogenic fronts and mesenchymal cells of the sutures. In particular, Boston-type craniosynostosis result from a single substitution of histidine for a proline in the N-terminal region of the Msx2 homeodomain (Jabs et al., 1993), a region that has been implicated in protein–DNA and protein–protein interactions (Vershon and Johnson, 1993). The molecular consequence of these mutations has been shown to alter the DNA-binding properties of Msx2 (amounting to a gain-of-function or constitutive activity) in such a manner that Msx2 has an enhanced affinity for its downstream target genes (Ma et al., 1996).

E. Craniofrontonasal Dysplasia and EphrinB1

Craniofrontonasal syndrome (CFNS) is an X-linked developmental disorder. Females have frontonasal dysplasia (an abnormality in the form of a frontonasal prominence) and coronal craniosynostosis (a fusion of the coronal sutures); in males, hypertelorism is the only typical manifestation. The classic female CFNS phenotype is caused by heterozygous loss-of-function mutations in EphrinB1 (Efnb1; Twigg et al., 2004).

Ephrins and Eph receptors are membrane-bound proteins that function as a ligand–receptor pair (Davy and Soriano, 2005; see Chapter 22) Upon binding, signaling can occur in both the forward (i.e., in the cell that expresses the Eph receptor) and the reverse (i.e., in the cell expressing the Ephrin ligand) directions, thereby providing a molecular conduit for cross-talk among tissues (Davy et al., 2004; Holder and Klein, 1999; Poliakov et al., 2004). In mice, the orthologous Efnb1 gene is expressed in the frontonasal neural crest, and it demarcates the position of the future coronal suture. Loss of Efnb1 disturbs tissue-boundary formation at the developing coronal suture. It is hypothesized that a patchwork loss of Efnb1in humans with CFNS disturbs tissue-boundary formation at the developing coronal suture, thereby resulting in craniosynostosis (Twigg et al., 2004).

VII. CONCLUSIONS

A. Development, Diversity, and Deformity

Despite the characteristic appearance of faces from different species, the structural edifice of the craniofacial complex is so highly conserved that its underlying pattern is shared by nearly all vertebrates. To the untrained (and perhaps even to the trained) eye, an early stage mouse embryo is indistinguishable from that of a human embryo. One might then wonder what forces act to establish this common craniofacial bauplan and what the driving forces are behind the divergence in craniofacial form among species. As Darwin and many other scientists speculated, the answer to both questions lies in natural selection. If natural selection guides these changes, how, then, are the molecular cues altered? Further, how do these molecular alterations result in morphologic change? New studies suggest that even slight variations in gene

or protein expression can lead to massive morphologic alterations. Future craniofacial studies will undoubtedly attempt to shed light on this issue to help elucidate both normal and aberrant craniofacial development.

SUMMARY

- The vertebrate face is derived from seven prominences. There are three paired prominences: (1) the lateral nasal prominences, which give rise to the alae of the nose; (2) the maxillary prominences, which gives rise to the upper jaw; and (3) the mandibular prominences, which give rise to the lower jaw. The lone single prominence—the frontonasal prominence—gives rise to the medial structures of the face, including the nose and the philtrum of the upper lip.
- The lower face and neck are derived from five pharyngeal arches (the first, second, third, fourth, and sixth).
- The first pharyngeal arch gives rise to the both the maxillary (upper jaw) and mandibular (lower jaw) prominences.
- The bones of the skull vary in origin; they can be either mesodermally or neural-crest derived.
- Cleft lip and palate are fairly common occurrences (1 in every 2500 live births) that result from either the failure of the palatine shelves to fuse, the inadequate migration and/or proliferation of cells, or the failure of the removal of the epithelial seam between the palatal shelves.
- HPE is a disease that is characterized by a wide continuum of craniofacial defects that range from cyclopia to severe brain anomalies accompanied by midline clefting to relatively mild cleft lip and palate. Microforms of HPE also exist, such as in cases in which the clinical manifestation of the syndrome is as subtle as a single central incisor tooth or close-set eyes. Some individuals with HPE can also have completely normal facial appearances. Genes within the Shh pathway, including the Hedgehog receptor Ptch and the downstream transcription factor Gli2, have been associated with HPE.
- Gain-of-function mutations within the FGF signaling pathway often result in craniosynostosis.
- Craniofrontonasal dysplasia is characterized by abnormalities in the frontonasal prominence and coronal craniosynostosis. Efnb1 has been genetically linked to this disorder. A heterozygous loss-of-function mutation in Efnb1 results in the characteristic phenotype of craniofrontonasal dysplasia by causing disruptions in the formation of tissue boundaries in the frontonasal prominence and the developing coronal suture.

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GLOSSARY

Chondrocranium
A subdivision of the craniofacial complex that contributes to the bones of the base of the skull and the bones that encase the sense organs.

Craniofrontonasal dysplasia
A rare disorder that is characterized by widely spaced eyes (hypertelorism); a flat, broad nose; and/or a vertical groove down the middle of the face; also known as *median cleft face syndrome*.

Craniosynostosis
The classification given to any condition resulting in the premature fusion of one or more of the cranial sutures.

Frontonasal prominence
The facial prominence that gives rise to the midline features, including the forehead, the middle of the nose, the philtrum of the upper lip, and the primary palate.

Holoprosencephaly
A disorder characterized by the failure of the prosencephalon (the embryonic forebrain) to develop. Holoprosencephaly is caused by the failure of the prosencephalon to divide to form bilateral cerebral hemispheres (the left and right halves of the brain), thereby causing defects in the development of the face and in brain structure and function.

Pharyngeal arches
A paired series of five bilateral outpouchings surrounded by an outer, surface ectoderm and an inner, pharyngeal endodermal that surround a mesenchymal core. The arches give rise to the structures of the lower half of the face and the neck; also referred to as *pharyngeal arches*.

Splanchnocranium
A subdivision of the craniofacial complex that includes the bones of the lower face and jaw.

Sutures
Fibrous membranes that exist between the flat bones of the skull.

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RECOMMENDED RESOURCES

USEFUL WEB SITES

Embryonic development:
http://www.med.unc.edu/embryo_images/unit-welcome/welcome_htms/contents.htm
Holoprosencephaly:
http://www.ninds.nih.gov/disorders/holoprosencephaly/holoprosencephaly.htm
Craniofacial anomalies:
http://www.faces-cranio.org

V

MESODERMAL ORGANS

31 INDUCTION OF THE CARDIAC LINEAGE

ANDREW S. WARKMAN and PAUL A. KRIEG

Department of Cell Biology and Anatomy, University of Arizona Health Sciences Center, Tucson, AZ

INTRODUCTION

The heart, which is a highly specialized muscular vessel, is among the first organs to develop in the vertebrate embryo. The heart of all vertebrates arises from symmetrically paired mesodermal tissues located on either side of the embryonic midline. As a result of the combined effects of gastrulation movements and tissue repositioning associated with embryonic morphogenic folding, the two heart patches move anteriorly and meet at the ventral midline, where they fuse to form a single cardiac primordium. By this stage, the precardiac tissue has already begun the expression of a number of heart muscle genes that serve as markers of cardiac differentiation. As development proceeds, the fused heart tissue folds upon itself to form a simple linear tube. The linear heart tube then undergoes a complicated rightward looping process that establishes the relative location of the atria and ventricles of the developing heart.

Classic embryological studies into early heart development have typically used chick and amphibian embryos; both of these embryos develop outside of their mothers, and embryonic tissues are extremely resilient to manipulations. More recently, powerful genetic methods have increased our understanding of heart development in mouse and zebrafish embryos, although, for practical purposes, these embryos are not suited for microsurgery or manipulation. Although the final structure of the heart is somewhat different in these different vertebrates (e.g., mouse and chick hearts contains four chambers whereas the frog and fish hearts contain three and two chambers, respectively) the fact that vertebrate hearts appear to be almost identical at the linear heart tube stage suggests that the initial events in heart formation are highly conserved. Through the study of these model systems, it has become apparent that commitment to the heart lineage in vertebrates involves a complex interplay of multiple tissue interactions that provide both inductive and inhibitory signals.

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Fate-mapping studies in the chick embryo indicate that the cells destined to become the heart are among the first cells to involute during gastrulation. Before gastrulation, these cells are bilaterally distributed in the epiblast lateral to the mid portion of the primitive streak. Explants of epiblast cells into culture media demonstrate that these cells are yet to be specified, because they are unable to differentiate into heart muscle (Holtzer et al., 1990; Yatskievych et al., 1997). At the onset of gastrulation (stage 3; Hamburger and Hamilton, 1951), cells fated to become heart cells are located within the anterior third of the primitive streak, excluding Hensen's node (Figure 31.1; Garcia-Martinez and Schoenwolf, 1993). Transplant assays of the precardiac mesoderm after it

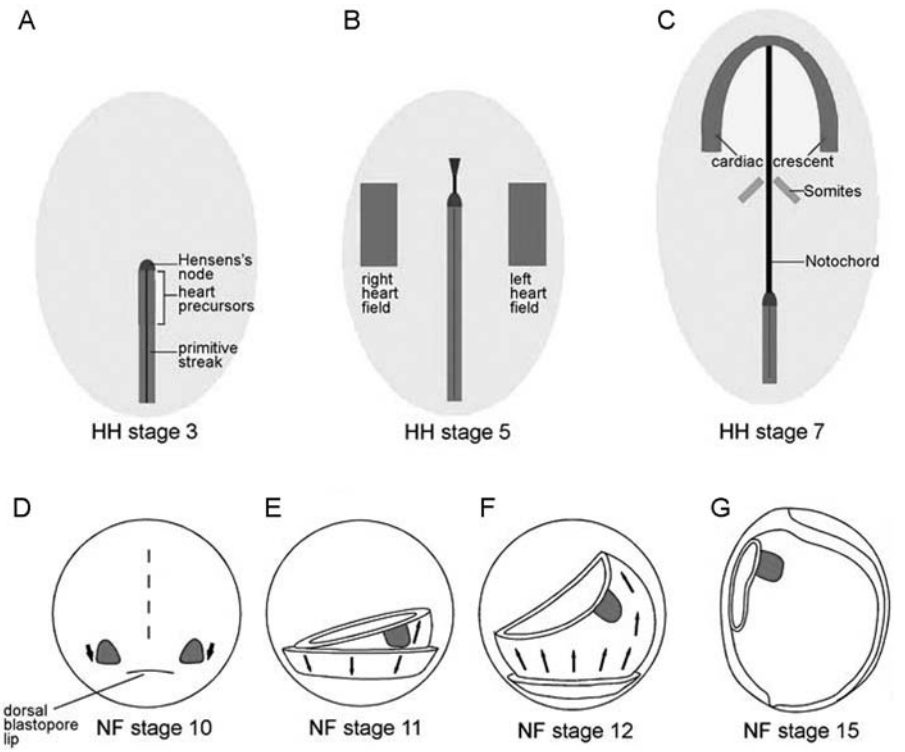

FIGURE 31.1 **Morphogenic movements associated with the developing heart in chick (A through C) and frog (D through G) embryos. A,** The heart precursors in the chick embryo at Hamburger and Hamilton (HH) stage 3 are first located within the anterior third of the primitive streak, just posterior to Hensen's node. Additional cells fated to form heart are present within the preinvolution hypoblast layer adjacent to the streak, but they are not depicted here for simplicity. **B,** As development proceeds, the cardiac precursors move through the streak and migrate anteriorly and laterally to form two heart fields on either side of the primitive streak (HH stage 5). **C,** By HH stage 7, the heart-forming region of the embryo has assumed a crescent shape after anterior–medial migration and the subsequent joining of the two heart fields at the anterior end. The folding of the embryo causes the heart fields to move medially, where they fuse and fold to form a linear heart tube. **D,** Dorsal view of early gastrula frog embryo. At the onset of gastrulation (Nieuwkoop and Faber [NF] stage 10), the heart precursors are located in bilateral patches on either side of the dorsal blastopore lip. **E** through **G,** Lateral views of the frog embryo (dorsal at right and anterior at top). **E** and **F,** After the involution movements of gastrulation, the heart primordia initially migrate anteriorly within the embryo. **G,** In the neurula embryo (NF stage 15), the two heart patches move ventrally, where they will fuse at the ventral midline and fold to form a linear heart tube. (Parts A through C are adapted from Brand [2003], with permission from Elsevier. Parts D through G are reprinted from Newman and Krieg [1999], with permission from Elsevier.)

has moved into the primitive streak and started to migrate anteriorly and laterally within the embryo (stage 4+ or 5) reveal that these cells appear to be specified for the cardiac lineage but not yet determined. Experimentally, this can be demonstrated by the observation that the precardiac mesoderm is able to differentiate into heart tissue when it is cultured in isolation (i.e., *in vitro* culture) but not when it is transplanted into the noncardiogenic regions of a similar-staged embryo (Antin et al., 1994; Gannon and Bader, 1995; Gonzalez-Sanchez and Bader, 1990; Montgomery et al., 1994). Interestingly, similar explant studies at later stages of development have demonstrated that mesoderm immediately medial to the cardiac mesoderm that does not normally contribute to the heart is able to give rise to cardiac tissue *in vitro* (Rawles, 1943; Rosenquist and De Haan, 1966). These were some of the earliest indications of a role for inhibitory signals in delimiting the size of the precardiac precursor population in the embryo. The heart precursors become committed to the cardiac lineage at around stage 6 (Montgomery et al., 1994), and the expression of terminal differentiation markers (e.g., myosin heavy chains) is first detected in the cardiac mesoderm at stage 7 (Han et al., 1992).

In Xenopus, the paired heart primordia are initially located on the dorsal side of the embryo on either side of the blastopore lip, and they are among the first mesodermal tissues to involute during gastrulation (see Figure 31.1; Keller, 1976). Studies of tissue explanted from the Xenopus early gastrula embryos (stage 10.5; Nieuwkoop and Faber, 1994) suggest that precardiac cells already exhibit some degree of specification before involution, because they are capable of differentiating into heart and other cells types in explant culture (Nascone and Mercola, 1995). Similar studies using late gastrula embryos (stage 12.5) demonstrate a higher degree of specification of the heart primordia, because explants exhibit robust cardiac differentiation in cell culture (Sater and Jacobson, 1989). It is also apparent that, similar to the situation reported for the chick, inhibitory signals play a role in delimiting the size of the heart primordia in Xenopus. Mesoderm cells from a stage 22 embryo that lie adjacent to the heart primordia but that would never normally contribute to cardiac tissue are capable of differentiating into cardiac cells in explant culture (Sater and Jacobson, 1990a). These results suggest that an inhibitory signal is preventing these tissues from differentiating into heart at their normal location in the embryo.

Classical studies of heart induction have commonly employed cut-and-paste experiments in which a naïve "responding" tissue is tested for its ability to react to inductive signals from an "inducing" tissue. The examination of the earliest events of cardiac induction in chick using blastula-stage embryos reveals that epiblast cells will give rise to heart tissue (among others) if they are cultured with underlying extraembryonic hypoblast tissue (Yatskievych et al., 1997). On the basis of these findings, it appears that the hypoblast provides an early general signal that serves to induce various mesodermal tissues, including heart. Similar studies have shown that the anterior but not the posterior endoderm from gastrulating chick embryos (stage 4 and 5 embryos) is able to induce cardiogenesis in posterior primitive streak explants that would not normally give rise to heart (Schultheiss et al., 1995). Finer mapping of the inductive properties of the anterior endoderm reveals that both the lateral and medial portions of this tissue exhibit inductive properties, although cardiogenesis *in vivo* occurs only in mesoderm that is associated with the lateral anterior endoderm (Schultheiss et al., 1995). Thus, it appears that the anterior endoderm generates a rather broad cardiac

field in the overlying mesoderm that is subsequently refined and restricted by additional signals.

Explant studies in Xenopus have suggested a requirement for the dorsal blastopore lip (i.e., the organizer) in the induction of heart from the precardiac mesoderm located on either side of the lip (Sater and Jacobson, 1990b). Unfortunately, the intimate association of the precardiac mesoderm with the underlying endoderm in the early Xenopus gastrula made the interpretation of these observations difficult. Moreover, these results appear to contradict studies in chicken embryos, in which organizer/node activity is not required for heart induction (Schultheiss et al., 1995). More recently, it has been shown that endoderm lying adjacent to the cardiac primordia in Xenopus gastrulae can function (albeit weakly) as heart-inducing tissue when it is cultured with precardiac mesoderm in the absence of organizer tissue (Nascone and Mercola, 1995). This result is more consistent with studies of the chick, in which anterior endoderm plays a central role in heart induction. So what role does the organizer play in heart induction? On the basis of the above observations, it has been proposed that the Xenopus organizer serves to initiate heart-forming competency within the flanking mesodermal tissue (Schneider and Mercola, 2001). After it has been rendered competent, mesoderm within the marginal zone is then able to respond to the heart-inducing signals provided by the endoderm. An alternate hypothesis—and by no means a mutually exclusive one—is that the organizer may serve to induce the production of a heart-inducing signal within the underlying endoderm; this in turn induces heart from the precardiac mesoderm adjacent to the organizer.

As mentioned previously, the mesoderm adjacent to heart primordia in chick and Xenopus embryos is able to give rise to heart in culture but not within the embryo. Explant studies using both amphibian and chick embryos have suggested the presence of a potent inhibitory signal to cardiogenesis within neural tissue (Climent et al., 1995; Garriock and Drysdale, 2003; Jacobson, 1961) and notochord (Schultheiss and Lassar, 1997). Overall, it is clear that both positive and negative signals resulting from various tissue interactions act in concert to induce cardiogenesis within a defined region of the developing embryo.

II. MULTIPLE SIGNALING PATHWAYS ARE INVOLVED IN EARLY CARDIOGENESIS

What is known about the signaling pathways that serve to regulate early cardiogenesis? The merger of classic embryology with modern molecular biology has led to the identification of numerous signaling molecules that appear to function within a regulatory network and that help to explain the complex interactions between the embryonic tissues that are required for the induction of the cardiac lineage (Figure 31.2). The sections below will provide a brief overview of the possible roles of different factors during early heart development.

A. Transforming Growth Factor-β Superfamily

1. Nodal Signaling

Cripto, which is the founding member of the EGF-CFC family of extracellular membrane tethered proteins, plays a central role in mediating nodal

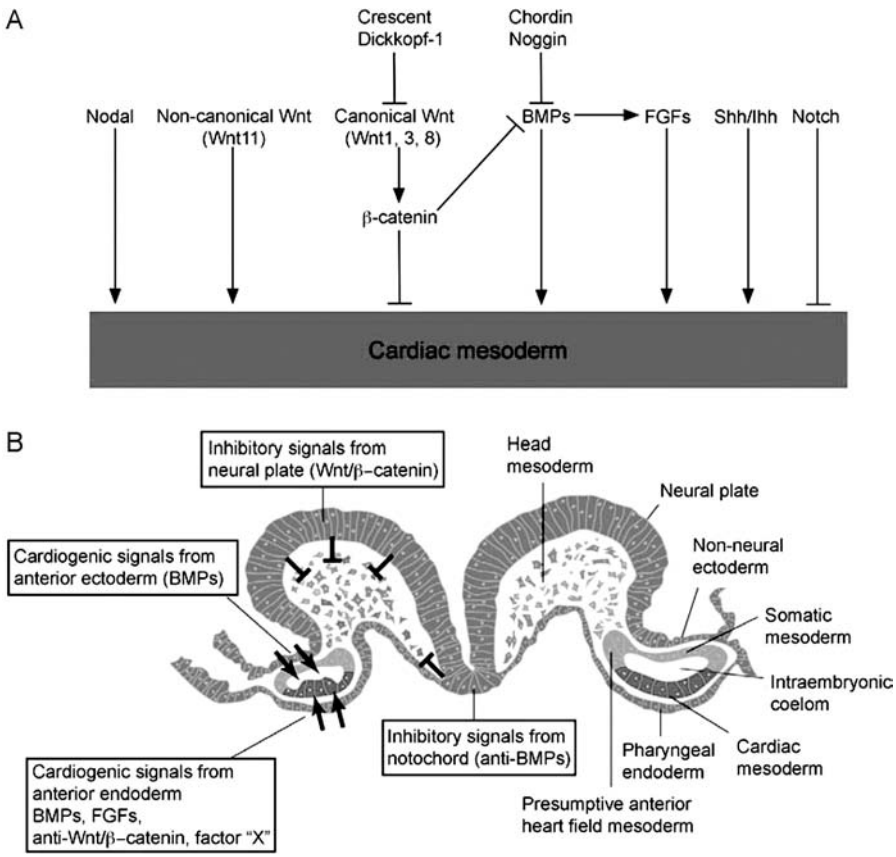

FIGURE 31.2 **Numerous signaling pathways serve to induce and restrict the cardiac lineage within mesodermal tissues. A,** An overview outlining the various signaling cascades and the interactions between them that play a role in vertebrate heart induction. **B,** A diagrammatic representation of a transverse section through the heart-forming region of a mouse embryo on embryonic days 7.5 to 8.0 illustrating the tissue interactions that generate both inductive and inhibitory cardiogenic signals. In each case, an arrow indicates a positive cardiogenic signal, whereas a bar denotes a negative cardiogenic signal. The term *factor X* is used to denote the yet-to-be-identified cardiogenic signal(s) that are present in the endoderm. (Part A is modified from Brand [2003], with permission from Elsevier. Part B is adapted from Solloway and Harvey [2003], with permission from the European Society of Cardiology). (See color insert.)

signaling by serving as an obligatory coreceptor (Yeo and Whitman, 2001). In the developing mouse embryo, Cripto is expressed in the nascent mesoderm, the developing heart field, and the linear heart tube (Dono et al., 1993). Analysis of the Cripto knockout mouse reveals that these animals die soon after gastrulation and that they contain a limited number of mesodermal cells. Although a number of precardiac transcription factors including Gata4, Mef2c, Hand1, and Hand2 are expressed, no transcription of cardiac differentiation markers is observed (Xu et al., 1999). Embryonic stem cells deficient in Cripto also exhibit an inability to express cardiac differentiation markers, although the expression of various cardiac transcription factors (e.g., Nkx2–5, Gata4, and Mef2c) appears to be unaltered (Xu et al., 1998). These findings represent one of the most dramatic phenotypes of all factors tested to date, and together they demonstrate a requirement for nodal signaling during the transition from cardiac specification to cardiac differentiation. Studies in zebrafish have

revealed that nodal signaling within the endoderm may also be required for proper cardiogenesis. For example, *one-eyed pinhead* mutant fish, which carry a mutation in a *Cripto*-related gene called CFC, exhibit a cardia bifida phenotype, presumably as a result of aberrant endoderm development (Reiter et al., 2001). Although the defects are clearly not as comprehensive as those observed in the Cripto knockout mouse, these observations provide additional support for the involvement of nodal signaling in early heart development.

2. Bone Morphogenic Protein Signaling

Studies in Drosophila have demonstrated an important role for the bone morphogenic protein (BMP)-related signaling protein decapentaplegic in the formation of the dorsal vessel (a structure that is broadly analogous to the heart in insects; Frasch, 1995). Studies in vertebrates have shown that at least three members of the BMP family are expressed within the cardiogenic region of a stage 5 chick embryo (Schultheiss et al., 1997). BMP-2 is expressed in the lateral endoderm, whereas BMP-4 and -7 are expressed in the ectoderm immediately adjacent to the precardiac mesoderm. Functional experiments using explant cultures have demonstrated that BMP-2 or -4 is able to induce cardiac differentiation and beating tissue when added to normally noncardiogenic tissue from anterior regions of the embryo. By contrast, the inhibition of endogenous BMP signaling in explants from the cardiogenic regions of stage 4 embryos results in a complete block of the expression of cardiac differentiation markers. In gain-of-function studies, the implantation of beads carrying BMP-2 or -4 *in vivo* was only able to induce cardiac differentiation in a limited region of the embryo (Schultheiss et al., 1997). Therefore, it is clear that the addition of BMP alone does not reflect the complete range of signaling activities at work in the intact embryo. Overall, these studies suggest that BMP signaling is necessary but not sufficient for the induction of the cardiac lineage.

Studies in other organisms have confirmed the importance of BMP signaling for cardiac development, although perhaps not at the level of cardiac specification. In Xenopus, BMP signals are essential for migration and/or fusion of the heart primordia and for cardiomyocyte differentiation as evidenced by the downregulation of the expression of Nkx2–5 and the reduced expression of myosin light chain-2 and cardiac troponin I (Shi et al., 2000; Walters et al., 2001). However, the fact that BMPs are not known to be expressed within the anterior endoderm or the organizer, in combination with the presence within the organizer of numerous BMP inhibitors, suggests that BMP signaling does not play a pivotal role in the earliest steps of heart induction in Xenopus. These observations can be reconciled if BMP signaling is necessary for later stages of heart development, after the heart primordia have assumed a more anterior position within the embryo (after gastrulation), but not for the initial specification of the cardiac lineage. The expression of Noggin (a potent BMP inhibitor) within the developing heart patches of both frog (Fletcher et al., 2004) and mouse (Yuasa et al., 2005) embryos provides further indication that the timing of BMP signaling is important and tightly regulated. Unfortunately, the genetic analysis of BMP signaling during early heart development in the mouse is complicated by the presence of functionally redundant activities. For example, the ablation of BMP-2 activity results in embryos with differentiated myocardial tissue but numerous structural malformations of the heart (Zhang and Bradley, 1996). The ablation of BMP-4 activity results in the death of the embryo before heart development (Winnier et al., 1995). Overall, although a role for BMP

signaling in the regulation of normal heart development is supported by experiments in all model systems, the precise role of BMPs during the induction of the cardiac lineage remains to be determined.

B. Fibroblast Growth Factor Signaling

The expression of fibroblast growth factors (FGFs)-1, -2, -4, and -8 in endodermal tissues of the stage 5 chick embryo places them in the correct place at the correct time to play a role in heart induction (Alsan and Schultheiss, 2002; Sugi et al., 1993; Zhu et al., 1996). Tissue explant assays demonstrate that FGF-4 in the presence of BMP-2 is able to induce cardiac differentiation in certain noncardiogenic tissues, whereas neither factor alone is sufficient (Lough et al., 1996). Moreover, the presence of a bead soaked in FGF-8 lateral to the heart field in chick embryos results in an expansion of the domain of expression of cardiac differentiation markers (Alsan and Schultheiss, 2002). Unfortunately, experiments using other model systems have not provided definitive evidence for the function of FGFs during heart induction. Mouse knockout studies show that both FGF-4 and -8 knockout embryos die early during gastrulation, which precludes the examination of their role in cardiac induction (Feldman et al., 1995; Sun et al., 1999). Alternatively, FGF-2 knockout animals are viable, with no obvious heart defects (Miller et al., 2000). In the chick embryo, the inhibition of FGF signaling using chemical inhibitors, neutralizing antibodies, and truncated FGF-receptor constructs does not appear to perturb heart induction (Alsan and Schultheiss, 2002). Taken together, these results suggest that FGF signaling is not sufficient for induction of the cardiac lineage. However, FGFs may cooperate with BMPs to promote heart induction and/or to regulate the size of the heart field.

C. Wnt Signaling

As mentioned previously, cardiac induction within the embryo appears to be molded at least in part by inhibitory signals emanating from the notochord and neural tissue. It seems likely that the inhibitory signal from the notochord involves the antagonism of BMP via the protein inhibitors Noggin and/or Chordin (Klingensmith et al., 1999). Alternatively, the inhibitory signal from the neural ectoderm may involve members of the Wnt family of secreted ligands. In the chick, at least two members of the canonical Wnt family that signal through β-catenin, Wnt1 and Wnt3a, are expressed in the dorsal neural tissue at the time of heart induction (Tzahor and Lassar, 2001). Ectopic expression of either of these molecules is able to mimic the repressive effects of neural tissue both *in vitro* and *in vivo*. By contrast, reducing Wnt activity in neural tissues by the ectopic expression of Wnt antagonists results in an expansion of cardiac tissues into areas that are not normally fated to form heart (Tzahor and Lassar, 2001).

The examination of endogenous Wnt inhibitors in the chick embryo has revealed that the Wnt antagonist, Crescent, is expressed in the anterior endoderm. Functional experiments using retroviral vectors to misexpress Crescent in the chick embryo result in an induction of cardiac tissue in regions of the embryo that do not normally contribute to the cardiac lineage (Marvin et al., 2001). These studies suggest that Crescent may play a central role in inducing cardiac tissue by repressing the canonical activity of Wnt3a and Wnt8c within the anterior portion of the chick embryo. Additional evidence for a Wnt repression mechanism of heart induction comes from studies using Xenopus. For

example, it appears that the endogenous Wnt antagonists, Crescent and Dickkopf-1, act to inhibit Wnt3a and Wnt8 activity originating from the organizer (Schneider and Mercola, 2001). This inhibition leads to the induction of precardiac tissue in the mesoderm on either side of the blastopore lip. By contrast, ectopic expression of either Wnt3a or Wnt8 was able to block cardiogenesis in tissues that would normally form heart (Schneider and Mercola, 2001). Although the inhibition of Wnt3a and Wnt8 activity appears to be required for heart induction in both Xenopus and chick embryos, it is interesting to note that the proposed source of the inhibitor signal differs in these organisms; Crescent and Dickkopf-1 are expressed in the organizer region of the frog embryo, but Crescent is expressed broadly within the anterior endoderm of the chick. Despite the differences in the domains of expression, both model systems suggest a single unifying mechanism: the blocking of canonical Wnt signaling leads to the induction of cardiac tissue in responsive mesodermal tissue. Perhaps the most dramatic demonstration of the importance of the inhibition of canonical Wnt signaling comes from mouse knockout studies, in which β-catenin activity was abolished in different embryonic tissues. When β-catenin expression was ablated in the embryonic endoderm, the resulting embryos formed numerous heart-like structures at ectopic locations (Lickert et al., 2002). Note that the knockout was in endoderm rather than mesoderm, which implies that still additional factors are likely to be involved in transmitting the cardiac induction signal to the precardiac tissues.

Other studies have suggested that signaling through noncanonical (i.e., non-β-catenin–dependent) Wnt pathways may be involved in cardiac induction. These studies have focused on Wnt11, which is expressed in the involuting mesoderm of the gastrula-stage frog embryo, including the precardiac mesoderm adjacent to the organizer. Studies in frog embryos (Pandur et al., 2002) reveal that the inhibition of endogenous Wnt11 signaling results in the decreased expression of the molecular markers associated with heart specification and differentiation and aberrant heart tube formation. By contrast, the ectopic activation of Wnt11 signaling is able to induce cardiac differentiation in regions of the frog (Pandur et al., 2002) and chick embryo (Eisenberg and Eisenberg, 1999) that do not normally form heart tissue. The interpretation of these results is complicated by the fact that the expression of high levels of noncanonical Wnt ligands appears to inhibit the canonical Wnt signaling pathway (Maye et al., 2004; Topol et al., 2003; Weidinger and Moon, 2003). Therefore, it is possible that the heart induction observed after Wnt11 overexpression is in fact mimicking the effects of the Wnt inhibitors Dickkopf and Crescent in inducing cardiac tissue. Certainly, the possible role of Wnt11 in cardiac induction is not shared in the mouse, where Wnt11 knockout animals form largely normal hearts (Majumdar et al., 2003).

D. Hedgehog Signaling

Gene ablation studies in the mouse indicate a role for hedgehog-signaling related heart development. Mice that are deficient for the hedgehog coreceptor protein, Smoothened, exhibit the delayed expression of the homeodomain transcription factor, Nkx2–5 (Zhang et al., 2001). Although Nkx2–5 expression does commence during later development (presumably as the result of activation through some other regulatory pathway), the linear heart tube fails to loop, thus indicating an essential role for hedgehog signaling in early cardiac

morphogenesis. Increased hedgehog signaling also causes heart defects. For example, the mutation of the inhibitory component of the hedgehog receptor, Patched, results in increased signaling by downstream components of the hedgehog pathway. Mice that are mutant for Patched show increased Nkx2–5 expression plus expansion of the expression domain. Although the exact nature of the regulatory interactions has yet to be resolved, it seems likely that cardiac progenitor cells come into contact with hedgehog signals originating from the node during gastrulation. Additionally, hedgehog signaling from the pharyngeal endoderm may also influence cells within the cardiac crescent during later development (Zhang et al., 2001).

E. Notch Signaling

In fish (Serbedzija et al., 1998) and amphibians (Sater and Jacobson, 1990a), the heart field appears to contain cells that will not normally contribute to the heart but that are capable of forming cardiac tissue if the definitive heart progenitors are removed. This regulative ability is gradually lost as development proceeds and eventually becomes restricted to the definitive cardiac precursors. Analysis in Xenopus of the mechanism behind this loss of plasticity has implicated Notch1 and its ligand Serrate (Rones et al., 2000). Increased Notch signaling results in the decreased expression of cardiac differentiation markers, whereas a reduction of Notch signaling using dominant-negative constructions results in increased myocardial gene expression. Studies in the chick have failed to reveal any regulative activity for the heart field, which suggests that this activity is not shared by all vertebrates (Ehrman and Yutzey, 1999).

III. TRANSCRIPTIONAL REGULATION DURING HEART INDUCTION

A. Hex

During Xenopus gastrulation, the homeodomain transcription factor Hex is expressed within the endoderm adjacent to the organizer (Jones et al., 1999). Hex is expressed in an equivalent domain within the anterior endoderm of the chick embryo, again in close proximity to the cardiogenic mesoderm (Yatskievych et al., 1999). These expression patterns place Hex in the correct place at the correct time to play a role in the cardiac induction pathway. Elegant studies in frog (Foley and Mercola, 2005) have confirmed a role for Hex at the earliest stages of heart induction. It appears that Hex regulates the production of a diffusible molecule within the endodermal tissue that induces heart in the adjacent precardiac mesoderm. Further analysis indicates that the expression of Hex in the endoderm is dependent on the generation of a canonical Wnt-free zone surrounding the organizer. This is achieved through the secretion of the Wnt inhibitors Crescent and Dickkopf-1 from the organizer itself. These findings provide a molecular basis for earlier observations that both endoderm and canonical Wnt inhibition are required for efficient cardiac induction in Xenopus. It seems likely that a similar mechanism is in effect during cardiac induction in chick, where Hex is expressed within the anterior lateral endoderm (Yatskievych et al., 1999) and canonical Wnt signaling is inhibited by Crescent. Mouse embryos mutant for Hex appear to undergo initial cardiac differentiation normally, although defects are observed in

cardiac morphology during later development (Hallaq et al., 2004). Therefore, it remains unclear whether an essential role for Hex in cardiac induction is shared by all vertebrates.

IV. TRANSCRIPTIONAL REGULATION IN PRECARDIAC TISSUE

The previous sections of this chapter have outlined how various tissue interactions serve to activate or inhibit the signaling cascades required for cardiac induction. However, the question of how these signals are ultimately deciphered within the precardiac mesoderm has yet to be addressed. The identification of transcription factors that are expressed within precardiac tissue has provided a valuable opening for examining the possible gene regulatory pathways lying downstream of the heart-inducing signals. The examination of the function of these genes, their expression in response to various signaling pathways, and the transcription-regulating elements in their promoters has generated significant insight into the regulatory networks that integrate cell signaling and ultimately give rise to the differentiated heart muscle.

A. The Nkx2 Family of Homeodomain Transcription Factors

In Drosophila, the homeodomain transcription factor tinman plays a central role in the initial formation of the dorsal vessel, a contractile muscular tube that is analogous to the vertebrate heart (Bodmer, 1993). Vertebrate genomes contain a number of *tinman*-related genes (reviewed in Evans, 1999; Firulli and Thattaliyath, 2002). The best-characterized one is called *Nkx2–5,* and it is expressed in precardiac tissues in all vertebrates examined. Additional genes related to *Nkx2–5* are present in all vertebrates, but the embryonic expression patterns of these genes vary, and the precise orthology among species is unclear. For example, the frog contains *Nkx2–3* and *Nkx2–10* genes, the chicken contains *Nkx2–3* and *Nkx2–8* genes, the zebrafish contains *Nkx2–3* and *Nkx2–7* genes, and the mouse contains *Nkx2–3* and *Nkx2–6* genes (Evans, 1999). The onset of Nkx2–5 expression in precardiac tissues during gastrulation appears to coincide with the timing of heart specification; however, the *Nkx2–5* gene of vertebrates does not function equivalently to the tinman gene of Drosophila. Unlike Drosophila tinman mutants, which express no heart markers, mice lacking Nkx2–5 function make a normal linear heart tube that expresses most cardiac differentiation markers, and embryos die as a result of severe defects in heart looping (Lyons et al., 1995). As a result of the expression of additional *Nkx2–5*–related genes in the heart, it seemed possible that the lack of a more severe heart phenotype in Nkx2–5 knockouts might be the result of functional redundancy. However, this does not seem to be the case, because Nkx2–5/Nkx2–6 double-knockout mice exhibit a phenotype similar to that of the Nkx2–5 knockout (Tanaka et al., 2001). Experiments in Xenopus show that Nkx2–5 expression is not sufficient to direct cells toward the cardiac lineage. The ectopic expression of either Nkx2–5 or Nkx2–3 in frog embryos caused an increase in the size of the normal hearts, but it was unable to induce the precocious expression of cardiac differentiation markers in the cardiac region or the ectopic expression of cardiac genes outside of the heart (Cleaver et al., 1996).

The regulation of *Nkx2–5* transcription in vertebrates is complex, and the gene is potentially downstream of numerous signaling pathways (Figure 31.3).

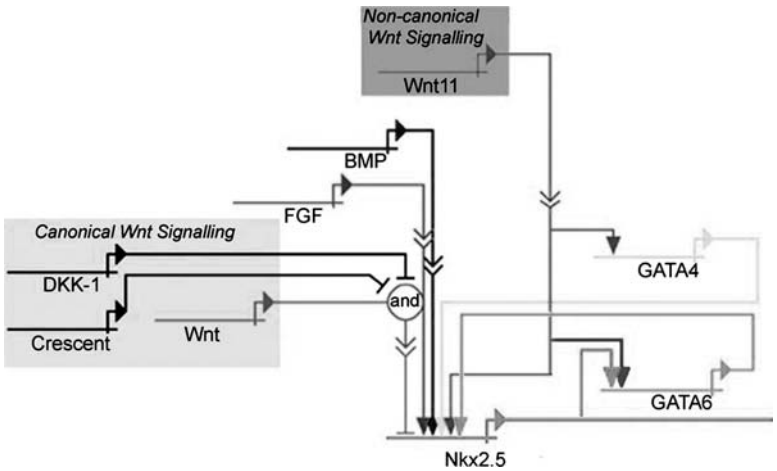

FIGURE 31.3 A regulatory network showing the intricate signaling cascades and feedback mechanisms that positively and negatively regulate (either directly or indirectly) the expression of the transcription factor *Nkx2–5* within the cardiac mesoderm. An arrow leading to the promoter of the *Nkx2–5* gene *(red)* represents a positive cardiogenic signal from the respective upstream gene, whereas a bar denotes a negative cardiogenic signal. A double chevron is used to indicate signaling that originates outside of the responding cell and that is mediated through its respective intracellular signaling cascade. (Modified from http://www.nottingham.ac.uk/biology/Genetics/staff/rogerpatient/networks/heart/heartnetwork.html. See color insert.)

Perhaps the most compelling evidence indicates that BMP is important for the early expression of Nkx2–5. Experiments in chick embryos demonstrate that BMP-4 can activate Nkx2–5 expression, even in noncardiogenic mesoderm (Schultheiss et al., 1997). In addition, transcription-regulating sequences of the *Nkx2–5* gene contain an evolutionarily conserved SMAD binding site that activates *Nkx2–5* transcription in response to BMP signaling (Liberatore et al., 2002; Lien et al., 2002).

B. GATA Family

The zinc finger transcription factor pannier is essential for heart development in Drosophila (Gajewski et al., 1999). The vertebrate homologues of pannier are the GATA transcription factors. In a way that is similar to Nkx2–5, vertebrate genomes encode a number of GATA transcription factors, three of which (GATA-4, -5, and -6) are expressed in the developing heart (reviewed in Peterkin et al., 2005). The analysis of the role of GATA factors in heart development is complicated by their overlapping expression domains and the fact that the factors are expressed in both the precardiac mesoderm and adjacent endodermal tissue. Mice that are deficient in GATA-4 exhibit cardia bifida, and they show a reduced number of cardiac precursor cells (Kuo et al., 1997) and the reduced expression of other precardiac genes, including Nkx2–5 (Reiter et al., 1999). Subsequent research has revealed that this phenotype arises as a result of a lack of GATA-4 activity in the endoderm rather than in the forming heart (Narita et al., 1997). GATA-5 knockout mice generate a normal heart (Nemer et al., 1999), which suggests that its function is not required during cardiac development. However, in zebrafish embryos mutant for GATA-5, the number of myocardial precursor cells is reduced, and so is the expression of several other cardiac factors, including Nkx2–5 (Reiter et al., 1999). GATA-6 knockout mice

die before heart induction as a result of a total lack of endoderm (Koutsourakis et al., 1999), and so the role of this factor in mouse heart development is unclear. The depletion of GATA-6 function in Xenopus embryos using antisense oligonucleotides results in decreased myocardial differentiation and an inability to maintain the expression of Nkx2–5 and Nkx2–3 in the developing heart (Peterkin et al., 2003). The ectopic expression of Xenopus GATA-4 in isolated ectodermal explants (i.e., animal caps) is able to induce beating tissue (Latinkic et al., 2003), whereas the ectopic expression of GATA-5 in zebrafish embryo results in the ectopic expression of Nkx2–5 and ultimately in the presence of clusters of beating cardiac cells (Reiter et al., 1999). Although the precise role of different GATA factors appears to vary in different organisms, the weight of evidence suggests that the expression of GATA and Nkx2–5 family proteins are closely linked in the cardiogenic tissue of the embryo. Indeed, it appears that GATA factors directly regulate the expression of *Nkx2–5* family genes and vice versa within precardiac tissues (Davis et al., 2000; Lien et al., 1999; Molkentin et al., 2000; Searcy et al., 1998). This interplay of transcription factors represents a potential mechanism for the amplification and stabilization of the cardiac transcriptional program in cardiac progenitor cells.

C. Islet-1

In mice, the transcription factor Islet-1 (Isl1) is expressed in various tissues within the developing embryo, including the foregut endoderm and a component of the anterior cardiogenic mesoderm (Cai et al., 2003). As the anterior precardiac tissues move to take up position in the developing heart, Isl1 expression is downregulated. Isl1 knockout mice die on embryonic day 10.5 as a result of severe cardiac defects, including the complete absence of outflow tract, right ventricle, and much of the atria (Cai et al., 2003). The mechanism underlying this dramatic phenotype appears to involve the aberrant proliferation, survival, and migration of cardiac progenitor cells. The expression of Isl1 within the foregut endoderm of mice raises the possibility that this gene may play a role in initiating or regulating the expression of the endoderm-derived heart-inducing signal (described previously) in the mammalian embryo, although this model has yet to be addressed experimentally.

D. T-box Transcription Factors

The T-box (Tbx) genes encode a large family of transcription factors, many of which play important roles in embryonic patterning and organogenesis. Clues to the involvement of Tbx factors in cardiac development came from the discovery that mutations in *Tbx1* and *Tbx5* genes were associated with DiGeorge syndrome (Chieffo et al., 1997) and Holt–Oram syndrome (Li et al., 1997), respectively, which are two common human congenital diseases that often exhibit heart malformations. The examination of the expression of *Tbx* genes has revealed that numerous Tbx family members are expressed in the cardiac mesoderm of vertebrates before cardiac differentiation (Plageman and Yutzey, 2005; Showell et al., 2006; Yamada et al., 2000). Knockout studies in mice suggest a high degree of functional redundancy between different family members, because the ablation of individual *Tbx* genes does not result in early cardiac defects. However, studies in Xenopus using a dominant-negative construction designed to inhibit general Tbx activity resulted in embryos with smaller hearts and, in some cases, even heartless embryos (Horb and Thomsen, 1999).

Determining the precise role of *Tbx* genes during early heart development is complicated by two additional factors. First, different members of the Tbx family act as transcriptional activators, whereas others are transcriptional repressors (reviewed in Plageman and Yutzey, 2005). Second, Tbx family proteins are capable of forming heterodimers, both with other Tbx proteins and other cardiac transcription factors, including GATA-4 and -5 and Nkx2–5 (Brown et al., 2005; Stennard et al., 2003).

E. Myocardin

Myocardin is a transcriptional coactivator of the ubiquitously expressed transcription factor serum response factor (Wang et al., 2001). In the mouse, myocardin expression commences in the developing heart at about the same time as Nkx2–5 expression (slightly later in smooth muscle cells). Myocardin knockout mice die early during development (on embryonic day 10.5) as a result of a vascular deficiency associated with a failure of smooth muscle development (Li et al., 2003). The presence of other myocardin-related proteins (MRTF-A and -B) within the developing heart suggests that the lack of a cardiac phenotype in these animals may be the result of functional redundancy. In Xenopus, myocardin itself is the only member of the myocardin family expressed in precardiac tissue, and the knockdown of myocardin activity using antisense methods results in a dramatic reduction in the expression of cardiac differentiation markers and the severe disruption of heart tube morphogenesis (Small et al., 2005). On the basis of these results, it will be interesting to determine the cardiac phenotype of mice in which myocardin and MRTF-A and -B function have been ablated.

V. CONCLUSIONS

The conservation of the underlying mechanisms (i.e., cell signaling and transcriptional regulation) involved in heart induction has enabled researchers to further our understanding of this complicated process using a variety of animal models ranging from fruit flies to rodents. Although no animal model system is perfect, we have gained considerable insight into both human heart development and disease by capitalizing on the advantages that each model system provides. Although our understanding of this complicated process is far from complete, improved knowledge will ultimately lead to an increased ability to diagnose, prevent, and treat congenital heart defects and disease.

SUMMARY

- The process of heart induction appears to be highly conserved among vertebrates.
- The endoderm is important for heart induction in vertebrates. The details of the mechanism are poorly understood, but the transcription factor Hex appears to be involved.
- The size of the developing heart is regulated by inhibitory signals.
- Signaling pathways stimulating cardiac induction include factors from the Nodal, BMP, and FGF families.

- The localized inhibition of canonical Wnt signaling, which is regulated by endogenous Wnt antagonists, is required for heart induction.
- The inductive process initiates the expression of numerous transcription factors within the precardiac mesoderm; these in turn activate the expression of genes associated with heart muscle differentiation.

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GLOSSARY

Cardia bifida
The formation of two beating and looping heart tubes as a result of the improper fusion of the heart primordia.

Determined
Cells are considered to be determined if they are able to assume their natural fate when placed in an antagonistic environment. Therefore, determination represents a higher level of commitment.

Induction
The process by which one group of cells signals a second group in a way that influences their development. This is most probably achieved through signaling involving diffusible factors.

Specified
Cells are considered to be specified if they are able to assume their natural fate when placed in a neutral environment (e.g., tissue culture media).

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32
HEART PATTERNING AND CONGENITAL DEFECTS

JOHN W. BELMONT

Departments of Molecular and Human Genetics and Pediatrics, Baylor College of Medicine, Baylor University, Houston, TX

INTRODUCTION

Cardiac development is one of the most studied processes in classic embryology, and, perhaps as a result of its complex elegance, it occupies a special place the field. During the last decade, genetic analyses have identified growth and morphogenesis pathways that are essential to specific components of cardiac organogenesis (see Chapter 31). These studies are transforming our understanding of normal cardiac development, and they show the essential unity of mechanisms across a wide range of model organisms. The genetic control of heart development is also important to clinical medicine, because congenital cardiovascular malformations (CVMs) are exceptionally common among all populations, and genetic factors appear to play a prominent role. Unfortunately, there is a distinct lack of data from both normal and abnormal human embryos, and the inference of molecular mechanisms is made more difficult by the lack of direct methods for determining which embryonic developmental step(s) were disturbed in an affected individual. The origins of many CVMs are obscure in that they may be interpreted as arising either as a secondary consequence of an early event or from the direct disruption of a later and more specific process. Because genetic analyses can often proceed without *a priori* knowledge of molecular pathways, genetics is likely to play a central role both for research involving the causes of CVMs and for the diagnosis of individual patients.

I. GENETIC EPIDEMIOLOGY OF CONGENITAL CARDIOVASCULAR MALFORMATIONS

A. Prevalence

Congenital heart defects are among the most common of all medically significant birth defects, and they are a leading contributor to infant mortality in the

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United States (Hoffman et al., 2004). The causes of CVM are complex and heterogeneous. Numerous environmental and genetic factors have been implicated in heart defects. Environmental agents include congenital rubella infection, *in utero* exposure to retinoids, and maternal diabetes. Cytogenetic abnormalities, exemplified by trisomy 21, often cause CVMs, and single-gene disorders have also been identified (e.g., Noonan syndrome, Holt–Oram syndrome). However, together, these conditions account for less than 20% of CVMs, and the causative factors contributing to most cases are not known. The high birth incidence (0.5%-0.9%) together with the substantial sibling recurrence risk (1%-3%) has suggested the hypothesis that CVMs have a multifactorial cause. Supporting this supposition is the fact that a much larger number of infants have minor anomalies of the heart at birth (e.g., small atrial and ventricular septal defects) that are found if imaging studies are performed without regard to symptoms. Bicuspid aortic valve (BAV) is also a very common anomaly that does not cause symptoms during early life. BAV may be the most common clinically important congenital heart defect, with studies in healthy adults indicating a prevalence of 0.9%. BAV is an important risk factor for subacute bacterial endocarditis and late-onset aortic valve calcification/stenosis in adults (Ward, 2000). Patent foramen ovale has been considered a normal variant in that it may occur in up to 30% of the population, but it also has a strong association with migraine and stroke (Horton and Bunch, 2004). The public health significance of developmental anomalies of the heart is enormous.

B. Cytogenetic Abnormalities

Chromosomal abnormalities are an important cause of CVM, accounting for 12% to 14% of all cases. Approximately 50% of children with Down syndrome have cardiac malformations (especially atrioventricular canal; Freeman et al., 1998). Trisomy 13 (Patau syndrome), trisomy 18 (Edwards syndrome), and monosomy X (Turner syndrome) are common causes of severe CVM. A handful of submicroscopic deletion and duplication disorders are also important contributors (Table 32.1). These disorders most likely affect several contiguous genes; however, as is exemplified by the TBX1 gene in 22q11del syndrome (Botto et al., 2003), a single gene is often thought to be the major factor causing the cardiac developmental defect.

C. Mendelian Disorders

More than 200 syndromes and single-gene disorders have been associated with cardiac malformations. Mutations in more than 100 genes that are involved in these disorders have been identified (see Table 32.1). Most of these conditions cause complex phenotypes or syndromes such that the cardiac component is only one somewhat variable component. Examples include Noonan (PTPN11), Holt–Oram (TBX5), CHARGE (CHD7), and Char (TFAP2B) syndromes (Satoda et al., 2000; Vissers et al., 2004; Stennard and Harvey, 2005; Tartaglia and Gelb, 2005). A less-common scenario (so far) is the identification of Mendelian genetic disorders in which the cardiac phenotype is either isolated or defining. Examples include familial atrial septal defect, which is caused by a mutation in NKX2.5 (Schott et al., 1998; Elliott et al., 2003; McElhinney et al., 2003), Gata4 (Garg et al., 2003), and MYH6 (Ching et al., 2005) as well as familial calcific bicuspid aortic valve, which is

TABLE 32.1 Genes Involved in Syndromic and Non-Syndromic Cardiovascular Malformations in Humans

Class	OMIM‡	Gene	Disorder or Syndrome	Heart Defects
Contiguous Gene	609625	10q	10qdel	PDA, ASD
	607872	1p36	1p36del	TOF/PA,PDA, ASD, VSD, DCM
	600309	1p31-p21	AVSD1	AVSD
	115470	tetrasomy 22q	Cat Eye	TAPVR, TOF
	123450	5p15del	Cri-du-chat	VSD, PDA, TOF, PA, PS, DORV
	609029	der(22)	Emanuel	ASD, VSD, PDA, PS
	147791	11q23del	Jacobsen	VSD, HLHS
	182290	17p11.2	Smith–Magenis	ASD, VSD, TOF, MR, AS, TAPVR
	247200	17p13.3	Miller–Dieker Lissencephaly	ASD
	601803	tetrasomy 12p	Pallister–Killian	AS, VSD, PDA, CoA, ASD, agenesis of the pericardium
	179613	8dup/del	Rec8 syndrome	TOF, DORV, TA
	192430	22q11del	Velocardiofacial	VSD, TOF+/-PA, IAA(B), TA, DORV, PDA
	194050	7q13del	Williams–Beuren	SVAS, PPAS, CoA , VSD, AS
	194190	4p16.3	Wolf–Hirschhorn	ASD , VSD
Transcription factor, chromatin regulator, or nuclear	300215	ARX	Lissencephaly, X-linked, with ambiguous genitalia	VSD, PDA
	301040	ATRX	Alpha-thalassemia/mental retardation, X-linked	VSD
	214800	CHD7, SEMA3E	CHARGE	TOF, IAA(B), VSD, DORV+/-AVSD, TA
	180849	CREBBP	Rubenstein–Taybi	VSD, ASD, PDA, CoA, HLHS
	166780	EYA1	Otofaciocervical syndrome	TOF
	153400	FOXC2	Lymphedema–distichiasis	TOF, VSD, TAPVR
	607941	GATA4, MYH6	Familial ASD	ASD
	200990	GLI3	Acrocallosal, Pallister–Hall	VSD, PS, ASD
	601536	HOXA1	Athabaskan brainstem dysgenesis	TOF
	169400	LBR	Pelger–Huet anomaly	VSD
	275210	LMNA	Lethal tight skin contracture	PDA , ASD
	300000	MID1	Opitz	VSD, persistent LSVC, ASD, PDA, DORV

	164280	MYCN	Feingold	PDA
	122470	NIPBL	Brachman–De Lange	BAV, VSD, PS
	108900	NKX2.5	ASD with conduction defect	ASD, TOF, dextrocardia
	117550	NSD1	Sotos	ASD, VSD, PDA
	148820	PAX3	Waardenburg type III	ASD
	309500	PQBP1	Renpenning	ASD, dextrocardia
	218600	RECQL4	Baller–Gerold	VSD, ASD
	107480	SALL1	Townes–Brocks	VSD, TOF
	607323	SALL4	Duane radial ray	VSD
	212550	SIX6	Microphthalmia, isolated, with cataract type 2	VSD, PDA
	601349	SNX3	Microcephaly, microphthalmia, ectrodactyly of lower limbs, and prognathism	VSD
	206900	SOX2	Microphthalmia and esophageal atresia	VSD, PDA
	114290	SOX9	Campomelic dysplasia	Complex
	602054	TBX1	DiGeorge, conotruncal anomaly face	TOF, PA, IAA(B), RAA, DORV VSD, TA
	181450	TBX3	Ulnar-mammary	PS
	142900	TBX5	Holt–Oram	VSD, ASD, HLHS, TAPVR, TOF, DORV
	169100	TFAP2B	Char	PDA, muscular VSD
	608771	THRAP2, ZIC3	Transposition of the great arteries	TGA
	106260	TP73L	Ankyloblepharon-ectodermal defects-cleft lip/palate	VSD, PDA
	101400	TWIST	Saethre–Chotzen	VSD
	235730	ZFHX1B	Mowat–Wilson	VSD, PDA
	603693	ZFPM2/FOG2	TOF	TOF
	306955	ZIC3	Heterotaxy 1, X-linked	Dextrocardia, TGA, PS, VSD, TAPVR, HLHS, CoA
Ligand, receptor, signal transduction	602730	ACVR2B	Heterotaxy	HLHS, AVSD, LSVC
	300166	BCOR	Microphthalmia, syndromic Type 2	ASD , VSD , MVP
	178600	BMPR2	Primary pulmonary hypertensions with CHD	AVSD, ASD, VSD, PDA, PAPVR
	605376	CFC1	Heterotaxy 2	DORV, TA, TGA, heterotaxy
	277300	DLL3	Jarcho–Levin	ASD, DORV

(Continued)

Table 32.1 Genes Involved in Syndromic and Non-Syndromic Cardiovascular Malformations in Humans—Cont'd

Class	OMIM‡	Gene	Disorder or Syndrome	Heart Defects
	305400	FGD1	Aarskog–Scott	ASD, VSD, PS, AS, CoA
	207410	FGFR2	Antley–Bixler	ASD
	101200	FGFR2	Apert	VSD
	218040	HRAS	Costello	HCM, PS, ASD, other valve dysplasia, dysrhythmias
	300472	IGBP1	Corpus callosum, agenesis of, with mental retardation, ocular coloboma, and micrognathia	VSD, PDA
	118450	JAG1, NOTCH2	Alagille	TOF+/-PA, CoA, PS, ASD, VSD
	115150	KRAS, BRAF, MEK1, MEK2	Cardiofaciocutaneous	ASD, PS, HCM
	603037	LEFTY1	Heterotaxy	Heterotaxy, HLHS, AVSD, LSVC
	259770	LRP5	Osteoporosis-pseudoglioma syndrome	VSD
	162200	NF1	Neurofibromatosis Noonan, Watson	PS, VSD, CoA
	109198	NOTCH1	Familial calcific bicuspid aortic valve	BAV, MS, VSD, TOF
	194200	PRKAG2	Wolff–Parkinson–White	Accessory conduction pathways
	255960	PRKAR1A	Intracardiac myxoma	ASD
	153480	PTEN	Bannayan–Zonana	ASD
	163950	PTPN11, KRAS	Noonan (NS1), LEOPARD	PS, HCM, CoA, ASD
	268310	ROR2	Robinow, brachydactyly type B1	PS, PA
	609192	TGFBR2, TGFBR1	Loeys–Dietz	PDA, ASD, BAV
	149000	VG5Q	Klippel–Trenaunay–Weber	PDA, ASD, PS, MVP
Structural or cell adhesion	267750	COL18A1	Knobloch	PDA, VSD, TAPVR
	200610	COL2A1	Achondrogenesis type II	ASD, AVSD
	606217	CRELD1	AVSD2	AVSD, heterotaxy, PA
	606617	DTNA	Left ventricular noncompaction	LSVC, PDA, HLHS
	185500	ELN1	Familial supravalvar aortic stenosis	SVAS
	608328	FBN1, ADAMTS10	Weill–Marchesani	AS, MI, PS, PDA, VSD
	121050	FBN2	Congenital contractural arachnodactyly	ASD, VSD
	309350	FLNA	Melnick–Needles	TOF
	150250	FLNB	Larsen	ASD, VSD
	312870	GPC3	Simpson–Golabi–Behmel type 1	VSD, PS, TGA, PDA, HCM

Metabolic	608688	ATIC	IMP cyclohydrolase deficiency	ASD
	602398	DHCR24	Desmosterolosis	TAPVR, PDA
	270400	DHCR7	Smith–Lemli–Opitz	AVSD, ASD, VSD, PDA, HLHS, CoA, PS, TAPVR
	608799	DPM1	Congenital disorder of glycosylation type 1e	PDA
	212066	MGAT	Congenital disorder of glycosylation, type IIa	VSD
	308050	NSDHL	Congenital hemidysplasia with ichthyosiform erythroderma and limb defects	ASD, VSD, single ventricle, CoA, Shone complex
	214100	PEX genes	Zellweger	VSD, PDA, HLHS
Ion channel	601005	CACNA1C	Timothy	VSD, TOF, PDA, long QT
	170390	KCNJ2	Andersen	BAV, CoA, long QT
DNA repair	227650	FANC genes	Fanconi	VSD, TOF
Monocilia	209900	BBS genes	Bardet–Biedl	VSD, Dextrocardia
	270100	DNAH11, DNAI1, DNAH5	Situs inversus viscerum, Kartegener	TGA, TA, VSD, ASD
Other or unknown	268300	ESCO2	Roberts, SC phocomelia	PS, PA
	225500	EVC,EVC2	Ellis–Van Creveld	Common atrium, AVSD, HLHS
	604896	MKKS	McKusick–Kaufman	TOF
	249000	MKS1	Meckel	ASD, VSD, CoA , PDA
	105650	RPS19	Diamond-blackfan anemia	VSD
	243800	UBR1	Johanson–Blizzard	ASD, VSD, dextrocardia
	273395	ZMPSTE24	Tetra–Amelia	PDA , ASD

[‡]Online Mendelian Inheritance in Man (http://www.ncbi.nlm.nih.gov/entrez/).

ASD, Atrial septal defect, primum or secundum; AS, aortic stenosis; AVSD, atrioventricular septal defect; BAV, bicuspid aortic valve; CoA, coarctation of the aorta; DCM, dilated cardiomyopathy; DORV, double outlet right ventricle; HCM, hypertrophic cardiomyopathy; HLHS, hypoplastic left heart; IAA(B), interrupted aortic arch type B; LSVC, persistent left superior vena cava; MVP, mitral valve prolapse; PA, pulmonary atresia; PDA, patent ductus arteriosus; PPAS, peripheral pulmonary artery stenosis; PS, pulmonic stenosis; Shone complex, parachute mitral valve, aortic stenosis, coarctation; SVAS, supravalvar aortic stenosis; TA, truncus arteriosus; TAPVR, total anomalous pulmonary venous return; TOF, tetralogy of Fallot; VSD, ventricular septal defect

caused by a NOTCH1 mutation (Garg et al., 2005; Garg, 2006). A third category involves genes that were initially identified as causing syndromic CVM but that have also been shown to play a role in patients with isolated heart defects. Examples include JAG1, which was originally identified in Alagille syndrome (Oda et al., 1997) but which was also found in subjects with apparently isolated Tetralogy of Fallot (TOF; Eldadah et al., 2001), and ZIC3, which causes X-linked heterotaxy (Ware et al., 2004) but which has also been observed in familial isolated transposition of the great arteries (TGA; Megarbane et al., 2000). Table 32.1 provides a list of human disorders and their associated genes. The list is meant to convey the range of defects seen rather than a quantification of which heart defects are most common or characteristic. An important point is that not all defects with the same name are mechanistically the same, with ventricular septal defect (VSD) and atrial septal defect (ASD) being glaring examples. Greater descriptive precision and hierarchical classification systems may better delineate the anatomic commonality within molecularly defined syndromes. Because many of these disorders are very rare, it may not be possible to know the true phenotype spectrum. Mechanisms that lead to cardiac phenotype heterogeneity in these disorders include the following: (1) allelic heterogeneity; (2) epistasis (i.e., gene interactions with modifier loci); (3) gene–environment interactions; (4) molecular pleiotropy (i.e., molecular pathways act as cassettes that get used repeatedly in different developmental stages, such that the gene products play more than one role in discrete embryologic processes); and (5) early developmental disturbance could lead to various anatomic outcomes influenced by mainly random processes.

These same factors contribute to the apparent complex inheritance of CVM. Familial aggregation, higher offspring recurrence risk as compared with sibling recurrence risk (Burn et al., 1998), and stable rates of CVM across time and in various populations are strongly suggestive of the idea that genetic factors are important. Heritability estimates (the relative contribution of genetic factors) are high but probably underestimated: (1) CVM are approximately 10-fold more common in miscarried pregnancies, which indicates that many affected offspring may be unobserved; and (2) affected individuals have reduced reproductive fitness. A spectrum of causal genes and mutant alleles is predicted by standard population genetics theory. Moreover, reduced reproductive fitness of individuals with CVM is predicted by the theory of selection to reduce the frequency of causative alleles across all involved genes. The most extreme scenario is that most CVM arise as the result of mutations unique to a single family, with their relative frequency being mainly the result of the large number of genes that play a role in normal cardiac development. This could be called the "large mutational target" hypothesis. However, even a weakly penetrant gene variant may contribute a substantial part of the overall population risk if it were common (Pritchard and Cox, 2002). At this time, a model involving both rare and common variants—possibly with gene and environmental interactions—is the most likely.

II. HEART TUBE

The commitment of precardiac mesoderm is covered elsewhere in this book and will not be reviewed here (see Chapter 31). Human heart development

begins at day 18 as the cardiogenic mesoderm coalesces toward the midline. Splanchnic mesenchyme aggregates to form a pair of endothelial tubes that fuse in the midline to form a single heart tube. As the heart tubes fuse, splanchnic mesenchyme proliferates and forms a myoepicardial mantle. The future cardiac myocytes are separated from the endothelial lining of the heart tube by cardiac jelly, which is a gelatinous connective tissue matrix. The Wnt inhibitory molecules, crescent and dickkoph, are expressed by the cardiac mesoderm, and the ectopic expression of these in posterior mesoderm is sufficient to induce heart formation (Marvin et al., 2001). Cardiac induction is mediated in part by bone morphogenetic proteins (BMPs)-2, -4 and -7 produced by adjacent neuroectoderm and foregut endoderm. In mice, the cardiogenic mesoderm expresses the transcription factor NKX2.5, a homeobox family member with specific functions in both early and late cardiac development (Patterson et al., 1998; Prall et al., 2002). NKX2.5 is the vertebrate homologue of Drosophila tinman, which is required for the formation of the heart-like vessel in flies. Another important family of transcription factors—Gata4, Gata5, and Gata6—are all expressed in the precardiac mesoderm (Patient and McGhee, 2002). Mutants in Gata4 exhibit a failure to fuse the bilateral heart tubes. By day 22, the single heart tube elongates, and segmentations marked by slight constrictions define the truncus arteriosus, the bulbus cordis, the primitive ventricles, the atrium, and the sinus venosus. These indicate the anterior-to-posterior patterning in the otherwise symmetric heart tube. At the anterior end, the truncus arteriosus is continuous with the aortic sac and aortic arch arteries. At the posterior end of the heart tube, the sinus venosus receives the umbilical, vitelline, and common cardinal veins from the chorion (primitive placenta), yolk sac, and embryo proper, respectively. Shortly after coalescence into this heart tube, rhythmic contractions occur. The heart tube is sensitive to flow in that normal flow is required for its proper growth and remodeling. Experimental obstruction to flow leads to morphologic abnormalities that are suggestive of human outflow defects like coarctation of the aorta (CoA) and hypoplastic left heart syndrome (HLHS; Hove et al., 2003).

Concomitant processes are involved in specifying chamber identity. The transcription factors Mesp1 and Mesp2 play a cell-autonomous role in ventricular but not atrial chamber formation. Irx4 is a homeodomain transcription factor that is highly expressed on the outer curvature of the heart tube where there is rapid growth and remodeling. Irx4 seems to be regulated by NKX2.5 and another transcription factor, dHand (Yamagishi et al., 2001). dHand (right) and eHand (left) exhibit generally complementary patterns of expression, and both are required for normal ventricle growth.

III. LOOPING

A. Left–Right Axis Patterning and Looping

The arterial and venous ends of the heart tube are fixed by the branchial arches and the septum transversum. Because the bulbus cordis and the outer curvature of the right ventricle grow faster than the other segments, the heart tube bends ventrally. This proceeds to a rightward folding of the ventricular segment forming the C-looped heart. As growth continues, an S-shaped heart

forms as the atrium and sinus venous are pushed dorsal to (i.e., behind) the bulbus cordis, truncus arterious, and future ventricles. The relative positions of the future left and right ventricles are also established at this stage. The direction of looping is controlled by underlying left–right (LR) patterning in the cardiac mesoderm. There are several linked processes that mediate this patterning: (1) LR symmetry breaking; (2) formation of the node (an "organizer" tissue), which will relay LR positional information to the lateral plate mesoderm (LPM); (3) expression of the Nodal-dependent signal transduction pathway in the left LPM; (4) stabilization by the expression of additional morphogens of the lateralized patterning in both the left and right LPM; and (5) transfer of positional information to organ primordia. The node is a critical organizer that forms at the anteriormost portion of the primitive streak (Brennan et al., 2002). The node is composed of specialized epithelial cells with ventral monocilia. Some of the monocilia exhibited gyrorotatory motion, and that led to the "nodal flow hypothesis," in which the motion of the monocilia generates a morphogen gradient that induces left-side identity. This mechanism has been further reinforced by the observation of membrane-bound "nodal vesicular packages," which contain Sonic hedgehog (Shh) and retinoids (Tanaka et al., 2005; Hirokawa et al., 2006). The production of nodal vesicular packages was shown to require fibroblast growth factor 8 signaling and to induce intracellular calcium transients in target tissue. These mechanisms seem to converge on the induction of Nodal expression, a transforming-growth-factor-β–family signaling molecule that plays a variety of roles in the early embryo (see Chapter 14). Nodal is a ligand for specific receptors with intrinsic serine threonine kinase activity. The activation of nodal receptors depends on membrane-bound coreceptors that then trigger the phosphorylation of Smad2/3, releasing it from the inhibitory Smad and allowing for interaction with Smad4. Phospho-Smad2/3 translocates to the nucleus, where it interacts with both inhibitory and activating cotranscription factors TGIF and FAST1. These in turn activate Nodal, Lefty-2, and PITX2 expression. The establishment of a midline barrier to left signals crossing into the right involves the prechordal plate and the notochord. These structures are dependent on the dorsal–ventral patterning of Shh, and mutations in Shh lead to left isomerisms as a result of the failure of the midline. Lefty1 is a nodal antagonist that is expressed in medial left LPM that is required to delimit the area of nodal activity, and mutations lead to left isomerism. The transfer of positional information to organ primordia is mediated in part by PITX2, a homeobox transcription factor that plays a central role in cardiac growth and the development of the common outflow tract.

B. Heterotaxy

Heterotaxy or situs ambiguus means discordance in the relationship between the normally asymmetric organs of the thorax and the abdomen. Heterotaxy arises from abnormal LR patterning with abnormal symmetry or reversals of cardiac chambers, vessels, lungs, and/or abdominal organs. An affected individual may have segmental discordances (e.g., TGA), loss of structures (e.g., asplenia), improper symmetry (e.g., right atrial isomerism in which left atrial development is concomitantly lost), or failure to regress symmetrical embryonic structures (e.g., persistent left superior vena cava). Heart defects typically combine malposition or TGA, ASD, VSD, persistent left superior vena

cava, anomalous pulmonary venous return (TAPVR), double-outlet right ventricle (DORV), common atrium, atrioventricular septal defects (AVSDs), pulmonary atresia and stenosis, CoA, or HLHS. Dextrotransposition (d-TGA) must be distinguished from levotransposition (l-TGA; also called *congenitally corrected transposition*) in that the latter implies a leftward looping of the heart tube at the C-loop stage (Figure 32.1). Left-looping clearly involves a disturbance in early LR axis patterning. The result is discordance of the outflow tract with the ventricles (i.e., morphologically, the right ventricle receives oxygenated blood and pumps to the systemic circulation via the aorta). If there is also atrioventricular discordance (i.e., left-atrium-to-right-ventricle connection), then the result is called *ventricular inversion.* The embryologic mechanisms underlying d-TGA are ambiguous in that d-TGA is frequently seen with LR patterning defects, but it may also arise from abnormal outflow tract septation (described later).

More than 80 genes associated with laterality defects in animal models and a small number of single-gene disorders have been identified in humans.

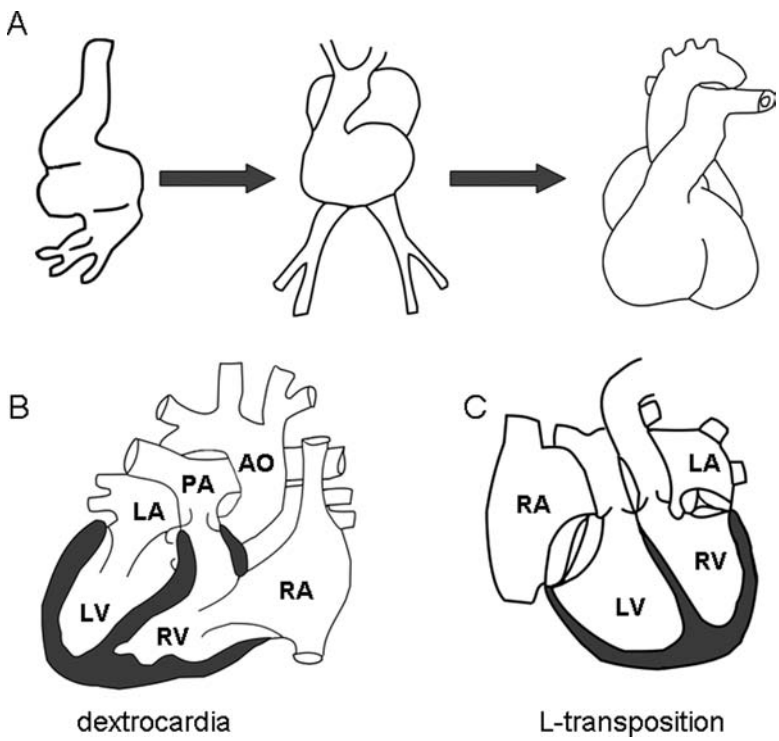

FIGURE 32.1 Heart tube and looping stage defects. A, The primitive heart tube is initially symmetric but undergoes rightward looping to form the C-loop stage heart. Later, dorsal movement of the atria and differential growth of the ventricle leads to the final anatomic position of the atria rostral to the ventricles. **B,** Dextrocardia results from complete reversal of the LR axis so that all of the heart structures are concordant. *LA,* Left atrium; *LV,* left ventricle; *RA,* right atrium; *RV,* right ventricle; *PA,* pulmonary artery; *Ao,* aorta. C, L-Transposition (sometimes called *corrected transposition*) also results from leftward looping, but there is discordance of the ventricles and atria. Note that the anatomic right atrium connects to the anatomic left ventricle, which pumps blood to the pulmonary artery. The corresponding atrioventricular valve has two leaflets. The anatomic left atrium connects to the anatomic right ventricle, which acts as the pump for the systemic circulation through the aorta.

Abnormalities in monocilia presumably explain the association of heart defects with primary ciliary dyskinesia caused by mutation in DNAH5, DNAH11, and DNAI1. The Bardet–Biedel syndromes, which have a surprising degree of locus heterogeneity, are caused by mutations in the genes required for cilia assembly or regulation, and this could explain the occasional patient with Bardet–Biedel syndrome having dextrocardia or heterotaxy. Heterotaxy and related isolated congenital heart defects have been associated with mutations in ZIC3, ACVR2B, LEFTYA, and CFC1; in some cases, this is supported by the observation of families with multiple affected individuals segregating specific mutations. All of these genes are known to be functionally connected to the Nodal signaling pathway, and, indeed, functionally deleterious mutations have been found in Nodal itself (unpublished observations). A number of other anecdotal observations of dextrocardia caused by mutations in very diverse genes such as PQBP1 (Renpenning syndrome, UBR1 [Johanson–Blizzard syndrome]), NKX2.5, and CRELD1 require further research, but they could suggest that diverse insults to the early cardiogenic mesoderm might result in abnormal looping of the heart tube.

IV. ATRIAL SEPTATION

A. Endocardial Cushions and Atrial Septation

Partitioning of the future atria and ventricles in the right and left chambers begins about the middle of the fourth week. Areas of thickening of the subendocardial tissue on the opposing dorsal and ventral walls are called the *endocardial cushions*. By the fifth week, the endocardial cushions fuse in the midline to divide the atrioventricular canal into right and left partitions that will ultimately form the mature atrioventricular valves. Two transcription factors, PITX2 and FOG-2 (Svensson et al., 2000; Tevosian et al., 2000), are required in the formation of the atrioventricular canal, and their deficiency leads to abnormal atrial septation. FOG-2 specifically interacts with Gata4, and mutations in it lead to TOF in mice and humans. Atrioventricular canal defects are also observed in Tolloid-1 mutants (Clark et al., 1999).

At the level of the common atrium, the septum primum grows from the dorsal-anterior wall of the atrial chamber (Figure 32.3). The foramen primum is formed by the gap between the septum primum and the endocardial cushions. Before this gap is completely closed, perforations appear in the anterior central septum primum and coalesce to produce the foramen secundum. Late in the fifth week, the septum secundum emerges from the ventral anterior wall of the common atrium to the right of the septum primum. The septum secundum grows toward the endocardial cushions and thereby covers the foramen secundum. The septum secundum is incomplete, leaving an opening between the left and right sides called the *foramen ovale*. The upper part of the septum primum regresses from the anterior wall of the left atrium so that the remaining part of the septum primum forms a flap valve for the foramen ovale. Before birth, the foramen ovale lets most of the blood entering the right atrium from the inferior vena cava across to the left atrium. After birth, the foramen ovale fuses, and the atrial septum is complete. About 30% of normal individuals retain a patent but valve-competent foramen vale.

B. Atrioventricular Canal Defects

There are two broad classes of ASD: isolated ASD and AVSDs (Figures 32.2 and 32.3). Defects in the septum secundum, called *secundum ASD,* are the most common form, but abnormal valve-incompetent foramen ovale called *primum ASD* are also important. AVSD include a family of malformations that involve the inferior atrial septum and the superior ventricular septum. These have also been called endocardial cushion defects (see Figure 32.2). This class of anomalies is characteristic of Down syndrome. Mutations in CRELD1 have been associated with nonsyndromic ASD, but the mechanistic basis for this and confirmatory animal models are not yet available (Robinson et al., 2003). A locus for autosomal-dominant familial AVSD has been mapped to chromosome 1p31-p21, but a specific gene has not yet been identified. Given that the formation of the atrioventricular canal occurs very early in cardiac morphogenesis, it is not surprising that AVSD has been observed in heterotaxy (LEFTY1 and ACVR2B), in CHARGE syndrome caused by mutations in CHD7, and in families with mutations in NKX2.5. Common atrium is another early defect in atrial septation that at least secondarily features failure of the growth of both the septum primum and the septum secundum. This anomaly is characteristic of the Ellis Van Creveld syndrome caused by mutations in either EVC or EVC2. Atrioventricular canal and common atrium may be observed in Smith–Lemli–Opitz syndrome, a condition that results from defective cholesterol biosynthesis. The complex developmental defects are thought to result from a lack

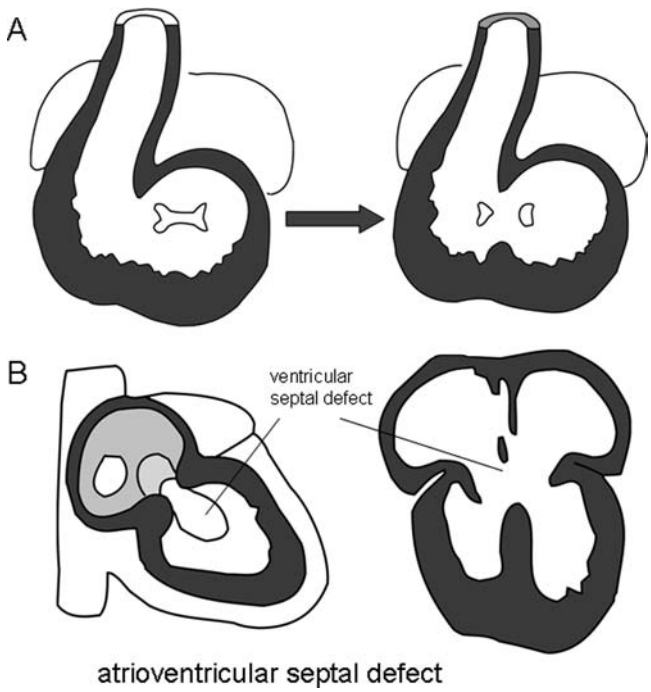

FIGURE 32.2 **Formation of the atrioventricular canal. A,** Before the partitioning of the primitive ventricle in the right ventricle and the left ventricle, blood flows from the common atrium through the atrioventricular canal. Growth from the endocardial cushion and differential growth of the ventricular myocardium lead to the formation of the ventricular septum. See Figure 32.3 for a sagittal view of the endocardial cushion. **B,** Failure of the complete closure of the atrioventricular canal leads to atrioventricular septal defects. (See color insert.)

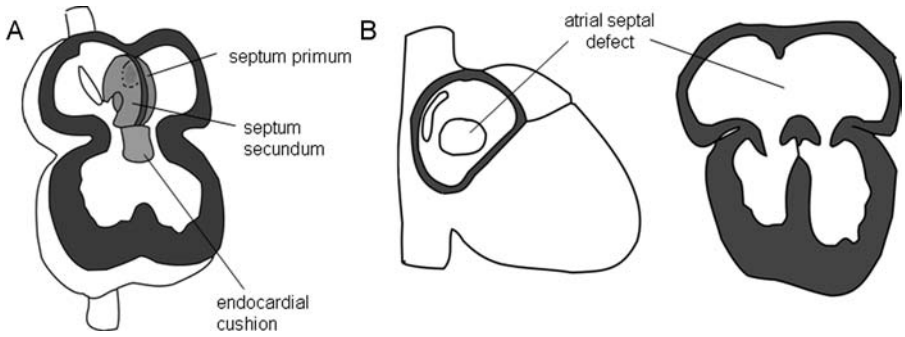

FIGURE 32.3 **Atrial septation. A,** Sequential growth of the septum primum and the septum secundum leads to the partitioning of the common atrium into the left and right atria. **B,** The failure of the closure of the septum leads to atrial septal defects. (See color insert.)

of cholesterol modification of Shh, which is another key signaling molecule and pathway. The very diverse defects associated with this condition are striking, and they strongly suggest very early abnormalities in heart development.

C. Atrial Septal Defects

Holt–Oram syndrome is the prototype of genetic disorders that cause ASD. This condition, caused by mutations in the transcription factor TBX5 (Basson et al., 1997; Stennard and Harvey, 2005), is almost always associated with thumb, hand, or radial malformations. During the past few years, additional ASD families have been identified, with mutations in another key cardiac transcription factor, Nkx2.5. These individuals often have associated cardiac conduction defects. Mutations in yet another key cardiac transcription factor, Gata4, were also found in a few families with isolated ASD, and, recently, mutation in the MYH6 gene has been observed in familial ASD. MYH6 encodes a structural protein that is a transcriptional regulatory target of TBX5, thereby establishing a potential functional connection between the two genetic disorders (Ching et al., 2005). ASD is a common associated finding in many other Mendelian disorders, which suggests that many different pathways are involved in atrial septation or that ASD is a relatively nonspecific outcome of early disturbances in cardiac development.

V. LEFT ATRIUM AND PULMONARY VEINS

A. Targeted Growth of the Pulmonary Vein Complex

As the lung develops, the primitive pulmonary venous plexus coalesces into the pulmonary veins, which in turn merge into the common pulmonary vein. It grows toward the primitive left atrium, where it fuses and is gradually incorporated into the wall of the left atrium. Later, the proximal branches of the pulmonary veins are incorporated into the dorsal wall of the chamber, and this results in four pulmonary veins with separate openings into the atrium. The left auricle, which is a remnant derived from the primitive atrium, develops trabeculations that reflect its distinct embryonic origin (as compared with the remainder of the smooth-walled left atrium). No mutations in animal models have yet been observed to specifically affect this interesting process.

B. Anomalous Pulmonary Veins

Although families have been found to segregate an autosomal-dominant, TAPVR-linked chromosome 4, to date, no gene identification has been reported (Bleyl et al., 1995). TAPVR and partial anomalous pulmonary veins are frequently found in conjunction with left and right isomerism sequences, respectively, as illustrated by mutation in ZIC3. Interestingly, TAPVR has occasionally been observed in a variety of disorders that do not have an obvious connection to either targeted growth of the pulmonary veins or LR patterning, such as Holt–Oram syndrome (TBX5), NKX2.5 mutation, and Smith–Lemli–Opitz syndrome (DHCR7). This suggests that it could result as a secondary abnormality in cardiac looping or in other early atrial growth defects, and it could explain the association with AVSD in some cases (e.g., persistent pulmonary hypertension associated with mutation in BMPR2; Roberts et al., 2004). There is a specific association of TAPVR with cat eye syndrome (tetrasomy 22q usually as an isochromosome), but it is difficult to attribute this to a single gene.

VI. VENTRICULAR SEPTATION

A. Atrioventricular Canal: Ventricular Septation

The separation of the right and left ventricles begins with a muscular fold in the constriction between the primitive ventricles (interventricular groove). Active upward growth of the myocardium from this constriction and the folding of the heart tube forms the muscular interventricular septum, but the wall between the ventricles remains incomplete through the seventh week. The final closure of the interventricular septum is coupled with partitioning of the atrioventricular canal and the common outflow tract. Ridges from both the right and left side of bulbus cordis emerge, and these in turn fuse with the ridge produced by the endocardial cushions of the atrioventricular canal. The membranous interventricular septum derives from the right side of the endocardial cushions joining the aorticopulmonary septum (conus septum) and the muscular part of the interventricular septum. At the closure of the interventricular septum, the pulmonary trunk connects with the right ventricle, and the aorta connects with the left ventricle. Several transcription factors have been shown to play a role in ventricular septation, including retinoic acid coreceptor, retinoid X receptor-alpha (RXRα), transcriptional enhancer factor-1 (TEF1), and Sox4 (Ya et al., 1998).

B. Ventricular Septal Defects

VSDs—although, broadly, the most common of all heart malformations—are anatomically heterogeneous. Although the cardiology literature is clear about this, animal models and human genetic studies most often fail to make important distinctions about critical anatomic details. Perimembranous VSDs occur within and adjacent to the membranous septum (i.e., they are formed by the fusion of the endocardial cushion with the superior portion of the muscular septum). Perimembranous VSDs can be divided into three types: outlet, inlet (which are the AVSD type), and trabecular. Defects in the outlet septum are thought to be caused by the failure of the fusion of the conus septum (described

later). Inlet defects may be caused by the failure of the complete fusion of the right superior endocardial cushion with the muscular septum. Muscular defects in the trabecular septum are probably the result of excessive remodeling of the interventricular wall or of the inadequate merging of the medial walls.

Although VSDs are the most common severe cardiovascular malformation, they convey the least information about the underlying mechanism. VSDs have been observed in almost all genetic disorders affecting heart development. Some represent a continuum of defects of the common outflow tract, as in velocardiofacial syndrome. Other VSDs are presumably the result of defects in cardiomyocyte growth, as in Holt–Oram syndrome, or defects in cardiomyocyte remodeling and survival, as in left ventricular noncompaction (Ichida et al., 2001). VSDs may also commonly accompany more complex defects and thus in those cases only represent a secondary anatomic defect.

VII. ENDOCARDIUM AND VALVULOGENESIS

A. Semilunar and Atrioventricular Valves

The semilunar (aortic and pulmonary) valves develop from three ridges of the endocardial tissue at the orifices of the aorta and the pulmonary trunk. These swellings go on to form the three thin-walled cusps of each valve. The atrioventricular valves (tricuspid and mitral) develop similarly from the localized proliferation of subendocardial tissue around the atrioventricular canals. The atrioventricular valve leaflets share characteristics with cartilage as illustrated by the expression of aggrecan and Sox9. The mutation of mouse NFATc leads to defective semilunar valve formation (de la Pompa et al., 1998). Double-mutant Egfr/Ptpn11 mutation in mice also leads to defective semilunar valve growth (Chen et al., 2000). Heparin-binding epidermal growth factor (EGF) and betacellulin are activating ligands for EGF receptors (EGFRs). The mutation of these ligands leads to defective endocardial valve precursor growth. These results highlight the importance of EGFR activation in valvulogenesis. As expected, other pathways are also active. For example, defective valvulogenesis occurs in tumor necrosis factor-α–converting enzyme mutants. From cell culture experiments, it is known that BMP-2 is sufficient to induce Sox9 and aggrecan expression in valve precursor cells consistent with their cartilage-like character (Lincoln et al., 2006).

Primary defects that affect blood flow into either ventricle may result in secondary ventricular hypoplasia, as seen in chick, fetal lamb, and zebrafish models, where the restriction of left ventricle inflow produces a phenotype similar to HLHS. Mice with mutations in HAND2, Bop, neuregulin, ERBB2, ERBB4, and RXRα exhibit primary ventricular hypoplasia or reduced wall thickness. Mice heterozygous for eNos mutation demonstrate bicuspid aortic valves.

B. Right Ventricular Outflow Tract Obstruction

Abnormal development of the pulmonary valve often leads to the obstruction of flow from the right ventricle. The characteristic lesions are called *pulmonary stenosis* or *pulmonary atresia*. These lesions often occur in combination with other defects, and they are a component of TOF (consisting of VSD, overriding aorta, pulmonic stenosis, and right ventricular hypertrophy). The pulmonary valve dysplasia of Noonan syndrome is a well-known example

of a specific association in which mutations in the intracellular phosphatase PTPN11 lead to the constitutive activation of mitogen-activated protein kinase signal transduction pathways, presumably including the EGFR pathway (described previously). Similar mechanisms are likely operating in the Costello syndrome (HRAS) and the cardiofaciocutaneous syndrome (BRAF, MEK1, MEK2, and KRAS). The genes mutated in those diseases encode proteins that all play important roles in mitogen-activated protein kinase signaling. The velocardiofacial/DiGeorge syndrome (deletion of 22q11) gives another good example of aberrant developmental mechanisms leading to pulmonary valve stenosis, pulmonary atresia, or even absent pulmonary valve. The transcription factor TBX1 is largely determinative in this condition, and rare patients have been found with point mutations in that gene. Pulmonary stenosis is also commonly associated with complex cardiac malformations in heterotaxy, again representing a secondary abnormality or perhaps the pleiotropic activity of certain pathways at multiple developmental stages.

C. Left Ventricular Outflow Tract Obstruction

Left-ventricular-outflow-tract-obstruction–type CVMs (Figure 32.4) include aortic valve stenosis (AS), CoA, HLHS, complicated mitral valve stenosis with HLHS and CoA (Shone complex), and BAV. CoA, AS, and HLHS are the most

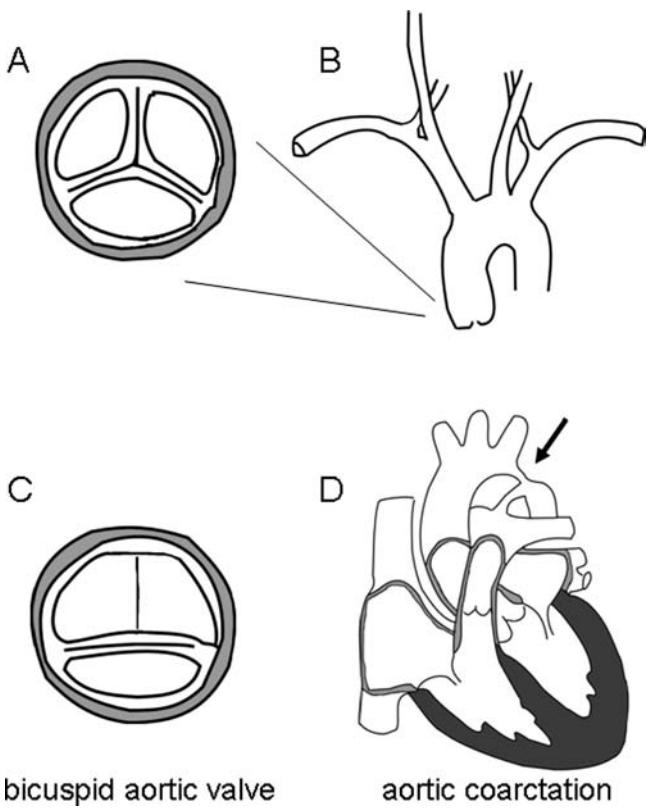

FIGURE 32.4 **The left ventricular outflow tract. A,** A normal, three-leaflet aortic valve. **B,** A normal aortic arch. **C,** The bicuspid aortic valve. Note the raphe in the upper leaflet, which indicates a fusion of the leaflets. **D,** Coarctation of the aorta *(arrow)*. The position of the narrowing is often at the ductus.

common CVMs seen in Turner (45,X) and Jacobsen (11q23del) syndromes. Several single-gene disorders are associated with left ventricular outflow tract obstruction defects, including Smith–Lemli–Opitz (DHCR7), X-linked heterotaxy (ZIC3; Ware et al., 2004), and Holt–Oram syndromes (TBX5). Multiplex families have been reported with CoA (MIM21000). Families with multiple occurrences of HLHS, AS, CoA, and BAV strongly suggest the existence of one or more discrete susceptibility genes being common to all of these defects (Ferencz et al., 1997).

VIII. COMMON OUTFLOW TRACT

A. Aorticopulmonary Septation

During the fifth week, ridges of the subendocardial tissue form in the bulbus cordis. Similar ridges also form in the truncus arteriosus, and they are continuous with those in the bulbus cordis. The spiral orientation of the ridges results in a spiral aorticopulmonary septum when these ridges fuse. This septum divides the bulbus cordis and the truncus arteriosus into two channels: the aorta and the pulmonary trunk. Blood from the aorta passes into the third and fourth aortic arch arteries (i.e., the future aortic arch), and blood from the pulmonary trunk flows into the sixth aortic arch arteries (i.e., the future pulmonary arteries). Several mouse mutants, including disheveled-2, semaphorin3C, and c-Jun, exhibit the failure of aorticopulmonary septation (Hamblet et al., 2002). Combinations of RARα1, RARβ, and RXRα gene mutations also result in muscular VSDs, DORV, arterial transposition, and truncus arteriosus (Lee et al., 1997). In addition, mutations in Fgf8 and TBX1 demonstrate common outflow tract developmental abnormalities that are suggestive of the type seen in DiGeorge syndrome. Mutations in endothelin, endothelin-A receptor, and ECE-1 lead to aortic arch malformations.

B. Neural Crest Contribution to Cardiac Development

At the time of the formation of the neural tube, neurectodermal cells at the dorsal ridge migrate into the embryo and differentiate into a wide variety of neural and mesenchymal cell types (see Chapter 26). Two broad domains of neural crest cells may be defined: cephalic and truncal. In addition, related cells emerge from the cephalic placodes and give rise to craniofacial structures such as the inner ear. The cardiac neural crest (CNC), which extends from the otic placode to the third somite, provides mesenchymal cells to the interventricular septum and the outflow tract. This same population of cells plays a critical role in the development of the thymus and the parathyroid glands. These cells transit through the third, fourth, and sixth pharyngeal arches. They participate in the secondary heart field related structures (described later), and they are required for the formation of the aorticopulmonary ridges. These cells also contribute to the walls of the aorta and the pulmonary arteries distal to the outflow tract. Various mutant models, including Pax3 (Conway et al., 1997), have been inferred to affect cardiac neural crest migration or differentiation. Pathways such as those requiring semaphorin3C (Feiner et al., 2001) are directly involved in controlling neural crest cell migration. Other pathways (as exemplified by the endothelin system, TBX1, and PITX2) may be involved in differentiation and growth or indirect mechanisms (Vitelli et al., 2002).

C. Anterior Heart Field

A second heart-forming field has recently been defined (Mjaatvedt et al., 2001; Kelly and Buckingham, 2002; Yutzey and Kirby, 2002), and it has been called the anterior heart field (AHF). It arises from a medial population of pharyngeal mesoderm cells just anterior to the cardiac crescent. Descendants of the precursors formed in the AHF give rise to the common outflow tract and the anterior structures of the mature heart, including the right ventricle and the proximal outflow tract, before the migration and differentiation of the neural crest. After that phase of AHF activity, the CNC cells interact with the mesoderm of the secondary heart field (SHF) to form the distal outflow tract (Kelly, 2005). Pharyngeal mesoderm cells of the AHF/SHF express transcription factors that are characteristic of cardiac mesoderm, including Nkx2.5, Gata4, MEF2C, and PITX2. PITX2 is also directly regulated by the Wnt/β-catenin pathway to control the development of the outflow tract via interaction with CNC cells (Kioussi et al., 2002). PITX2 is required by the SHF cells rather than the CNC, and its asymmetric expression (left is greater than right) may be an important factor is establishing correct arterial positioning and the spiral patterning of the conotruncal ridges (Ai et al., 2006).

D. Conotruncal Defects

Several common defects have their origins in the failure of the development of the common outflow tract (Figure 32.5). These include truncus arteriosus, TGA, DORV, TOF, and interrupted aortic arch type B. Truncus arteriosus represents a failure of aorticopulmonary septation, and, consequently, there is a single semilunar valve. As noted above, d-TGA may be associated with LR

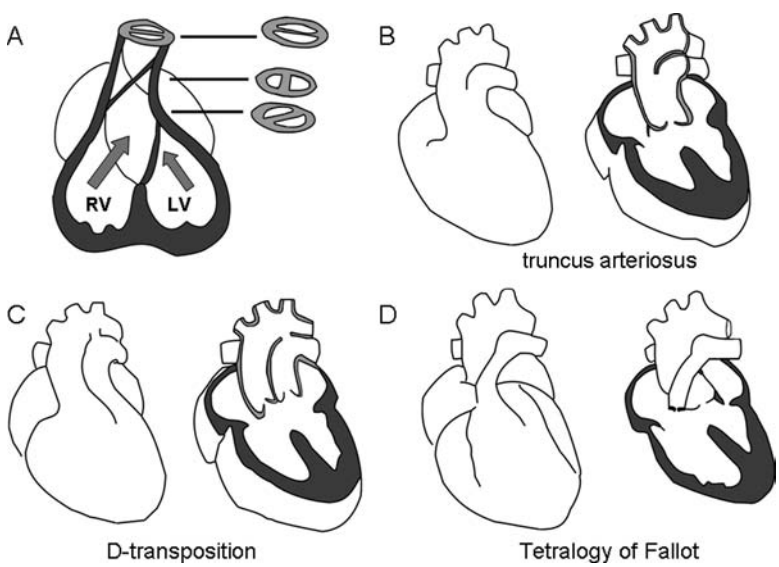

FIGURE 32.5 **The formation of the common outflow tract and aorticopulmonary septation. A,** Spiral growth of the conal ridges leads to the partitioning of the outflow tract to form the aorta and the pulmonary artery. **B,** The complete failure of the partition leads to truncus arterious. **C,** Anterior malpositioning of the aorta leads to the connection of the aorta to the right ventricle and of the pulmonary artery with the left ventricle. The resulting lesion can be either d-TGA or DORV (not depicted). **D,** Deficient growth of the proximal outflow tract leads to stenotic pulmonary valve *(arrow)*, VSD, and overriding aorta. This lesion is considered TOF.

patterning defects, but it may also arise from a much later failure of the conotruncal septum to spiral. That leaves the aorta anterior and rightward of the pulmonary artery, creating a connection of the aorta to the right ventricle and the pulmonary artery to the left ventricle. DORV is similar in that there is malpositioning of the aorta that causes it to receive flow from the right ventricle. Interrupted aortic arch type B refers to an interruption of the aorta between the takeoff positions of the carotid and subclavian arteries. It is understood to result from the failure of the development of the fourth and sixth aortic arch arteries, which specifically require the contribution of the CNC-derived cells arising from the secondary heart field. These defects seem to be part of a continuum, and variations are seen within specific genetic disorders. The velocardiofacial/DiGeorge syndrome (TBX1) is the prototypical conotruncal disorder. Similar heart defects are characteristic of the CHARGE (CHD7, SEMA3E) and Alagille (JAG1, NOTCH2) syndromes. JAG1 is a ligand for Notch-family receptors, and the finding of mutations in this gene in patients with Alagille syndrome and isolated TOF demonstrates an important role for that pathway in outflow tract development. The connection between these conditions is not at all clear, and, as yet, a functional interaction of Notch signaling with the TBX family of transcription factors has not been established.

E. Ductus Arteriosus

The ductus arteriosus is a normal structure that allows for the flow of oxygenated blood from the venous circulation to enter the systemic circulation in utero. After birth and the inflation of the lungs, the ductus closes, thereby allowing for the establishment of the separate venous and arterial circulations. The ductus arises from the left sixth aortic arch artery. Patent ductus arteriosus (PDA) results when the ductus fails to undergo its normal physiologic closure and involution. PDA is seen in numerous genetic disorders, and the causal mechanisms are very poorly understood. Char syndrome (TFAP2B) is an example of a relatively specific association in that other heart defects are not typically observed in that condition. However, PDA is also seen in such diverse conditions as Down syndrome, velocardiofacial syndrome, and many other much less common disorders (see Table 32.2).

TABLE 32.2 Acronyms of Cardiovascular Malformations

AS: Aortic stenosis
ASD: Atrial septal defect
AVSD: Atrioventricular septal defect
BAV: Bicuspid aortic valve
CoA: Coarctation of the aorta
DORV: Double-outlet right ventricle
HLHS: Hypoplastic left heart syndrome
LSVC: Left superior vena cava
PDA: Patent ductus arteriosus
PFO: Patent foramen ovale
PS: Pulmonary stenosis
SVAS: Supravalvar aortic stenosis
TA: Truncus arteriosus
TAPVR: Total anomalous pulmonary venous return
TGA: Transposition of the great arteries
TOF: Tetralogy of Fallot
VSD: Ventricular septal defect

IX. INTRACARDIAC CONDUCTION SYSTEM

A. Conduction System

At the heart tube and looping stages, the primitive common atrium acts as the pacemaker. As the chambers are formed, the atrioventricular constriction forms fibers that are specialized for conduction. The Purkinje fibers form the sinoatrial node, the atrioventricular node, and the atrioventricular bundle. They are innervated by making connections with neural-crest–derived autonomic ganglia that invade the subendocardial tissue. The sinoatrial node originates in the sinus venosus, later incorporating into the wall of the right atrium at the entrance of the superior vena cava. The atrioventricular node develops in the lower part of the interatrial septum. The atrioventricular bundle in the interventricular septum consists of Purkinje fibers that extend from the atrioventricular node to the ventricles. A specific role for Nkx2.5 in the development of the conduction system is demonstrated by both mouse and human mutations (Schott et al., 1998; Kasahara et al., 2001), and NKX2.5 null mouse embryos lack the primordium of the atrioventricular node (Jay et al., 2004). Connexin40, which is the major gap junction isoform of the Purkinje fibers, is likely to be a key downstream target of NKX2.5 as evidenced by the alteration of its expression with models of both overexpression and loss of function of NKX2.5 (Harris et al., 2006) . In addition, the mutation in the transcription factor HF-1β also leads to disrupted conduction system differentiation (Nguyen-Tran et al., 2000).

B. Familial Conduction Abnormalities

So far, at least three human disease genes have been associated with the abnormal development of the cardiac conduction system. The first such disorder to be discovered is caused by mutations in NKX2.5. Recently, a gene for familial Wolff–Parkinson–White syndrome was identified (Gollob et al., 2001). The fact that some individuals with this disorder also have cardiomyopathy suggests more complex abnormalities in the maintenance of cardiomyocytes. Tachyarrhythmias are also observed in some patients with Costello syndrome. This condition has been shown to result from mutations in HRAS (Aoki et al., 2005). Arrhythmias in each of these conditions are likely the result of either the abnormal development of or the subsequent loss of specialized conducting tissue within the heart.

SUMMARY

- There is now a remarkable opportunity for the application of information derived from basic molecular embryology to the broad challenge of human congenital heart malformations.
- The number of genes identified in Mendelian disorders with significant cardiac malformations has increased by at least 10-fold during the last decade.
- Using new methods in human genetics (e.g., comparative genome hybridization, dense single nucleotide marker panels, high-throughput DNA sequencing) may allow for both the testing of candidate genes identified in model systems and the identification of previously unsuspected loci.

- Experimental systems will play an essential role in determining how mutations and gene variants alter the normal network of growth, differentiation, and intercellular signaling required for normal cardiovascular development.

GLOSSARY

Cardiovascular malformation
Abnormality in the normal anatomy of the heart or major blood vessels resulting from disturbed development.

Coarctation of the aorta
Narrowing of the aorta that causes obstruction to blood flow.

Isochromosome
A chromosome that has lost one of its arms and replaced it with an exact copy of the other arm.

Tetralogy of Fallot
The combination of pulmonary valve stenosis, overriding aorta, ventricular septal defect, and right ventricular hypertrophy.

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RECOMMENDED RESOURCES

Online Mendelian Inheritance in Man:
http://www.ncbi.nlm.nih.gov/omim
Human Gene Mutation Database:
http://www.hgmd.cf.ac.uk/ac/index.php
Development of the Heart:
http://embryology.med.unsw.edu.au/Movies/heart.htm
Harvey RP, Rosenthal N, editors: *Heart development*, Burlington, MA, 1998, Academic Press, Inc.

33
BLOOD VESSEL FORMATION

KARINA YANIV and BRANT M. WEINSTEIN

Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD

INTRODUCTION

The development of the vascular system is one of the earliest events in organogenesis. All other organs depend on a vascular supply for the delivery of nutrients, oxygen, and cellular and humoral factors and for the clearance of wastes. Serious disruptions in the formation of the vascular network are lethal early during postimplantation development in amniotes, whereas the maintenance of vessel integrity and the control of vessel physiology have important consequences throughout embryonic and adult life. Many of the processes that take place during normal vascular development in the embryo are reactivated in situations of neoangiogenesis in the adult, including tissue regeneration, wound healing, and tumor formation. A full understanding of the signaling pathways of vascular development is essential when searching for new targets for therapeutic intervention during pathologic situations.

I. EMERGENCE OF THE BLOOD VASCULAR SYSTEM

A. Basic Concepts

The cardiovascular system consists of the heart, the blood vascular system, and the lymphatic vascular system, which is discussed separately later in this chapter. During vertebrate embryogenesis, it is the first functional organ system to develop, because embryonic growth and differentiation are critically dependent on the transport of oxygen, nutrients, and waste products throughout the early vasculature. The heart and blood vessels form a closed circulatory loop, with blood never leaving the vessels, except through leakage or hemorrhage. The vascular system is composed of two fundamental types of blood vessels: arteries and veins. Arteries carry blood away from the heart to tissues, whereas veins return blood back to the heart (except for pulmonary veins). The circulatory systems of fish, amphibians, reptiles, birds, and mammals show various stages of evolution. In fish, the system has only one circuit, with the blood being pumped

through the capillaries of the gills, where it is oxygenated and then sent on to the capillaries of the body tissues before being returned to the heart; this is known as *single* circulation. The heart of the fish is therefore only a single pump that consists of two main chambers: the atrium and the ventricle. In air-breathing vertebrates, an additional circulatory loop is incorporated to accommodate the pulmonary circulation; blood flows from the heart to the lungs for oxygenation and then back again to the heart before being sent out again for distribution to other tissues. In amphibians and reptiles, this double circulatory system is used, but the heart is not always completely divided into two separate pumps (e.g., amphibians have a three-chambered heart). Birds and mammals show complete separation of the heart into two pumps for a total of four heart chambers (two atria and two ventricles). Oxygen-saturated blood leaves the heart through the aortic arch, branching out and dispersing into ever-smaller–caliber arteries and arterioles. Eventually, the blood supply comes into contact with all of the living cells of the body in the capillaries, which are microscopic vessels through which life-sustaining substances and wastes move readily in and out. From the capillaries, blood moves into small venules and then progressively larger veins, which merge together into the vena cava before returning to the heart.

Blood vessels all have a similar basic histologic structure (Figure 33.1). They are composed of two basic cell types: vascular endothelial cells (ECs)

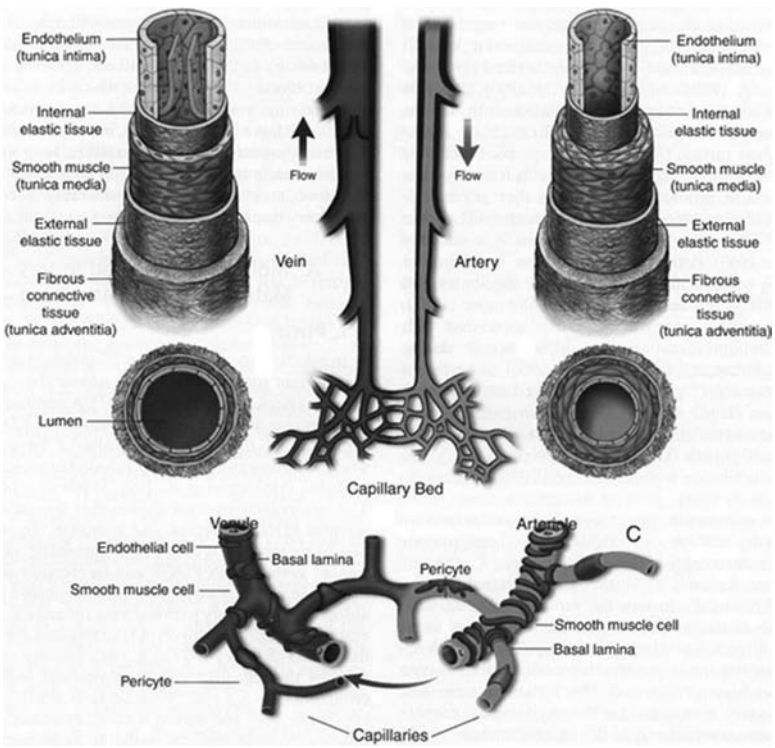

FIGURE 33.1 **Blood vessels come in two fundamental types: arteries and veins.** Both types of vessels are composed of an inner endothelium (tunica intima) surrounded by internal elastic tissue, a smooth muscle cell layer (tunica media), external elastic tissue, and fibrous connective tissue (tunica adventitia). Larger-caliber arteries have a thicker smooth muscle cell layer, whereas larger veins possess specialized structures such as valves. The two networks of tubes are completely separate at the level of the larger vessels but are connected distally through a system of fine capillaries. (Reproduced from Cleaver and Krieg, 1999, with permission. See color insert.)

and vascular smooth muscle cells (VSMCs). The inner epithelial lining of the blood vessels adjacent to the lumen is a thin, single-layered epithelium of ECs, whereas smooth muscle cells or pericytes surround the EC layer. In larger blood vessels, the inner endothelial lining is called the *intimal layer,* and it is surrounded by a *medial layer* that is composed of multiple layers of VSMCs embedded in an elastin-rich extracellular matrix. The medial layer is itself surrounded by an extracellular matrix-rich layer called the *adventitial layer.* The inner lining of the vessel is the endothelium, a single-cell–thick layer of vascular ECs that is surrounded by subendothelial connective tissue. This is surrounded by a muscular layer of VSMCs, which is highly developed in arteries. Finally, there is a further layer of connective tissue called the *adventitia,* which contains nerves that supply the muscular layer as well as nutrient capillaries in the larger blood vessel.

The earliest primitive vessels in vertebrate embryos form by a process called *vasculogenesis,* during which mesodermal cells differentiate into endothelial precursor cells called *angioblasts.* These angioblasts then differentiate in situ into ECs and coalesce to form the earliest vessels, which in mammalian and avian embryos often appear as a relatively unstructured vascular plexus. The subsequent remodeling of vasculogenic vessels and their further growth and remodeling to form the complex and elaborate network of vessels found in the mature vasculature is called *angiogenesis.* In nonsprouting angiogenesis or intussusception, preexisting vessels subdivide in two by the formation of transvascular posts or pillars, whereas, during sprouting angiogenesis, new vessels grow by sprouting and growth from preexisting vessels (see Chapter 21).

After the primitive endothelial tubes are formed, the endothelium secretes factors that lead to the recruitment and/or induction of primordial smooth muscle through a process called *vascular myogenesis.* Several recent reviews have carefully documented the current state of knowledge regarding the differentiation and growth of VSMCs to form the tunica media (Carmeliet, 2000; Hungerford and Little, 1999). The origins of VSMCs remain unclear. Different models have been proposed for the induction and differentiation of these cells (Bergwerff et al., 1998; DeRuiter et al., 1997; Rosenquist and Beall, 1990; Vrancken Peeters et al., 1999); however, it is important to note that the complex origin of VSMCs seems to be dependent on their location. This suggests that individual growth factors and their receptors will have different effects on VSMC growth and differentiation in specific vascular beds.

B. Emergence and Specification of Endothelial Cells

The initial phase of vascular development involves the differentiation of endothelial precursor cells, called *angioblasts,* from mesoderm. Angioblasts are endothelial precursors that have certain characteristics of ECs but that have not yet assembled into functional vessels (Flamme et al., 1997). Quail/chick transplantation experiments have shown that two subsets of mesoderm—somitic and splanchnopleuric—have the potential to give rise to endothelial progenitors in avians (Coffin and Poole, 1988; Pardanaud and Dieterlen–Lievre, 1999). In zebrafish embryos, angioblasts detected by the expression of vascular endothelial growth factor receptor (VEGFR-2/flk1/kdr) arise and segregate from the lateral plate mesoderm at the 7-somite stage (Fouquet et al., 1997; Liao et al., 1997; Thompson et al., 1998). Cell-lineage analysis

suggests that these angioblasts contribute to the primordia of the dorsal aorta, the cardinal veins, and even the intersegmental vessels in the zebrafish trunk (Childs et al., 2002; Zhong et al., 2001). In the developing mouse embryo, the formation of blood islands in the extraembryonic yolk sac marks the onset of vasculogenesis (Risau and Flamme, 1995). Blood islands develop from aggregates of mesodermal cells at approximately embryonic day 7.5 to 8 of mouse development. They consist of an inner layer of primitive hematopoietic cells and a peripheral population of angioblasts. These angioblasts differentiate into ECs, form a lumen, migrate, and interconnect to form a primary vascular plexus (Risau and Flamme, 1995). The close spatial relationship as well as the simultaneous emergence of the hematopoietic cells and ECs within the blood islands has led to the hypothesis that they arise from a common precursor, the hemangioblast (His, 1900; Murray, 1932; Williams et al., 1980; see Chapter 34).

Several lines of evidence support the existence of such progenitors with dual potentials. For example, angioblasts and hematopoietic progenitors express many of the same transcription factors and surface receptors, such as CD34 (Fina et al., 1990), Flk, Flt1, Tie1, Tie2 (Dumont et al., 1995), and SCL/tal–1 (Kallianpur et al., 1994). Moreover, the development of both endothelial and hematopoietic compartments is impaired in embryos bearing a mutation or a dominant-negative form of one of the relevant genes (Shalaby et al., 1995; Shivdasani et al., 1995; Visvader et al., 1998). Similarly, the zebrafish *cloche* mutation results in defects in blood cells and blood vessels (Liao et al., 1997; Stainier et al., 1995).

Probably the most compelling evidence for the hemangioblast, however, comes from studies involving the *in vitro* differentiation system of mouse embryonic stem cells, in which the development of the endothelial and hematopoietic lineages in embryoid bodies recapitulates events that take place *in vivo* in the yolk sac blood islands (Doetschman et al., 1985; Vittet et al., 1996; Wang et al., 1992; Wiles and Keller, 1991). Using this model system, Choi et al. (1998) and Chung et al. (2002) isolated a transient population of cells that expresses markers that are common to both cell lineages (SCL/tal–1, CD34, and Flk–1), and they determined that single cells could give rise to clones containing both hematopoietic cells and ECs. More recently, an $Flk1^{+}$ population of cells was identified in the posterior primitive streak of embryonic day 7 to 7.5 mouse embryos, thus providing further support for the existence of a common progenitor for hematopoietic cells and ECs *in vivo* (Huber et al., 2004).

Although many studies looking at morphology, gene expression, and mutant phenotypes support the concept of a common hematopoietic cell and EC progenitor in the yolk sac *in vivo*, other studies have argued against its existence. In the avian embryo, a clonal differentiation assay of VEGFR-2–positive cells sorted out from the very early blastodisc failed to give rise to mixed colonies of endothelial and hematopoietic cells (Eichmann et al., 1997). In addition, the lineage tracing of cells from the primitive streak to the yolk sac has failed to identify cells with endothelial and hematopoietic potential (Kinder et al., 2001). Finally, Ferkowicz et al. (2003) showed that hematopoietic and endothelial progenitors can be distinguished by their differential expression of CD41 as soon as they exit from the primitive streak.

The differences between these apparently contradictory facts still need to be resolved. Some of the discrepancies may result from differences in the

timing of commitment to the different lineages in various organisms. Another explanation may be that there are distinct populations of precursor cells, some of which have multilineage "hemangioblastic" potential and others that contribute only to single lineages. EC differentiation in the embryo proper does not occur in close association with hematopoiesis, except for on the floor of the aorta (reviewed by Jaffredo et al., 2005a), where the endothelial and hematopoietic clusters are present in close proximity. Recent data have suggested that the endothelium of this region might be hemogenic (i.e., capable of giving rise to definitive hematopoietic cells through an endothelial intermediate). However, other studies suggest that hematopoietic precursor cells originate from the surrounding mesenchyme and migrate through the aorta wall before subsequently entering the circulation (reviewed by Jaffredo et al., 2005a; 2005b). Further work allowing for the direct tracing of endothelial progenitors as well as additional molecular determinants of the hemangioblast will presumably shed additional light on the molecular pathways leading to hematopoietic cell and EC lineage differentiation.

C. *De Novo* Formation of a Primary Vascular Plexus: Vasculogenesis

After endothelial progenitors have been specified, they interconnect to form a dispersed capillary plexus that supports blood cell circulation and that matures into a vascular network by extensive pruning and remodeling. This process, which involves the differentiation of ECs from the mesoderm and is followed by their coalescence into tubes, is called *vasculogenesis* (Risau, 1997). Intraembryonic vasculogenesis is initiated in the cranial region of embryonic day 7.5 mouse embryos with the emergence of endocardial progenitor cells. Concomitantly, the aortic primordia first become discernible (Drake and Fleming, 2000). In the chick embryo, the dorsal aorta as well as several capillaries has differentiated by the time that a heartbeat begins at the 12-somite stage (reviewed by Eichmann et al., 2005). As is seen in other vertebrates, the first angioblasts in the zebrafish arise from lateral plate mesoderm. The angioblasts migrate to the trunk midline between the 10-and 15-somite stages and coalesce to form the primary axial vessels of the trunk (reviewed by Weinstein, 2002). As shown in a very detailed atlas of the blood vessels of the developing zebrafish (Isogai et al., 2001), the same primary vasculogenic vessels that establish the initial circulatory circuits within mammalian and avian embryos are present in the zebrafish, including the dorsal aorta and the posterior cardinal vein in the trunk and the internal carotid artery, the primordial hindbrain channel, the anterior cardinal vein, and the basilar artery in the head (Figure 33.2).

D. Remodeling and Maturation of the Vascular Plexus: Angiogenesis

After its initial establishment, the vasculogenic primary vascular plexus becomes extensively remodeled and elaborated on by subsequent angiogenesis. This angiogenic remodeling and growth are essential for tissue and organ growth and repair, and an imbalance in this process contributes to numerous malignant, inflammatory, ischemic, infectious, and immune disorders. Angiogenesis takes two main forms: sprouting and nonsprouting (reviewed by Scappaticci, 2002). Sprouting angiogenesis refers to the development of new blood vessels by budding and growth from preexisting vessels. It involves the proteolytic degradation of the extracellular matrix adjacent to an existing vessel,

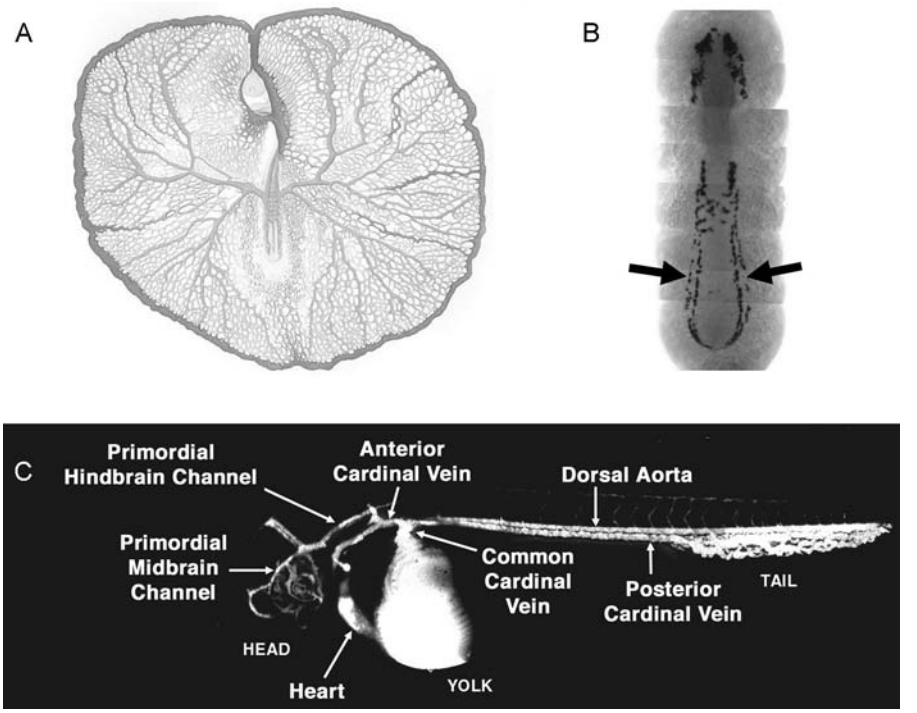

FIGURE 33.2 **Major primitive vessels form by vasculogenesis during embryonic development.** **A,** Early avian yolk sac vascular plexus, with larger arterial and venous vessels already apparent. **B,** In situ hybridization for the *fli1* gene *(arrows)* marks emerging vascular and hematopoietic progenitors in the early lateral mesoderm of the 10-somite–stage zebrafish *(composite image, dorsal view, anterior up)*. **C,** Early vasculogenic vessels in confocal microangiography of a 24-hour postfertilization zebrafish embryo. In fish and amphibians, most vasculogenic vessels form as single vascular tubes without an intermediate stage of vascular plexus formation. (Panel A modified from Popoff, 1894).

migration and proliferation of ECs from the wall of the vessel, followed by lumen formation and the maturation of functional capillaries from the mobilized ECs. Nonsprouting angiogenesis occurs by intussusception, during which the subdivision of previously existing vessels takes place by the formation of transvascular pillars that split the vessel into several new capillaries. Intussusceptive and sprouting angiogenesis are both employed to remodel, elaborate, and expand on an initial vascular plexus. This occurs concomitantly with the acquisition of VSMCs or pericyte cells, which stabilize the nascent vasculature and which are essential for the maturation phase of vessel development.

During organ and tissue growth, blood vessels must continuously grow and adapt to meet the needs for nutrients and oxygen. The organs and tissues signal to the vessels to promote their growth and, if necessary, their regression. They also provide cues that cause ECs to adopt functional specialties and the specific features that particular organs need to interact properly with the circulatory system (Nikolova and Lammert, 2003). It has become clear in recent years that ECs are functionally and molecularly heterogeneous. In addition to arterial–venous distinctions, ECs from different organs and tissues frequently express different genes. Conversely, increasing evidence suggests

that ECs, in turn, provide instructive morphogenetic cues to the surrounding organs to help determine their location, differentiation, and morphology during development and in the adult. Blood vessels and organ-specific cells interact with each other continuously throughout development and postnatal life, and the coordination between ECs and the cells within the organs and tissues that they serve generates a functional organ with a vascular system that is adjusted to its needs.

E. Molecular Regulation of Vascular Development

Vascular development and pathologic blood vessel formation are promoted by signals that are received and processed by ECs or their precursors. In recent years, a number of different signaling pathways involved in the development of the vascular system have been described (reviewed by Coultas et al., 2005). The best characterized involve receptor tyrosine kinases, although other classes of signaling inputs are also important (reviewed by Folkman and D'Amore, 1996; Ilan et al., 1998; Yancopoulos et al., 1998).

In the following sections, we review two pathways: the vascular endothelial growth factor (VEGF) and the angiopoietin/Tie receptor pathways, which are almost totally endothelial specific and which are critical players in vascular development. Other signaling factors and pathways that are important for the guidance and patterning of the developing vasculature are discussed in Section IV.

1. Vascular Endothelial Growth Factor Signaling

VEGF has been shown to be important for the migration, proliferation, maintenance, and survival of ECs, and it is critical during both vasculogenesis and angiogenesis (reviewed by Carmeliet and Collen, 2000; Yancopoulos et al., 2000). VEGF, which is now commonly referred to as *VEGF-A*, was the first growth factor described to be a mitogen specifically for ECs (Carmeliet and Conway, 2001; Ferrara, 1999; Ferrara et al., 2003). It was initially defined, characterized, and purified for its ability to induce vascular permeability and to promote EC proliferation. The VEGF family includes five characterized VEGF relatives in mammals (VEGF-A through VEGF-D and placental growth factor) that display differential interaction with three related receptor tyrosine kinases (VEGFR-1/Flt-1, VEGFR-2/Flk-1, and VEGFR3/Flt-4) and a number of ancillary receptor components, such as the neuropilins (NPs; reviewed by Goishi and Klagsbrun, 2004). VEGF-A signals through binding to VEGFR-1 and VEGFR-2, which are restricted largely to vascular endothelium in their expression, and this accounts for the specificity of VEGF-A signaling. By contrast, VEGFR-3 is restricted largely to lymphatic endothelium (Kukk et al., 1996). VEGF-A is produced by different cell types, including tumor cells, macrophages, T cells, and smooth muscle cells (Klagsbrun and D'Amore, 1996). It is thought to play a major role in tumor-induced neovascularization, and, recently, a humanized monoclonal antibody directed against VEGF-A has shown efficacy for the clinical treatment of colorectal and renal tumors (Willett et al., 2004). During embryonic development, the expression of both VEGF-A and its receptor VEGFR-2 correlate closely with sites of vessel formation (Jakeman et al., 1993; Liang et al., 1998; Shweiki et al., 1993).

The most conclusive evidence for the critical role of VEGF-A as a key regulator of both vasculogenesis and angiogenesis has come from the

knockout mice (Carmeliet et al., 1996; Ferrara, 1996; Shalaby et al., 1995). In embryos lacking either VEGF-A or VEGFR-2, blood islands, ECs, and major vessels fail to develop in appreciable numbers, resulting in embryonic lethality between embryonic days 8.5 and 9.5. Remarkably, the disruption of even one of the two VEGF-A alleles results in embryonic lethality between embryonic days 11 and 12, demonstrating a strict dose–dependent requirement for VEGF-A during embryogenesis and making this one of very few genes showing haploinsufficiency during murine development. The inactivation of VEGFR-1 also leads to embryonic lethality (Fong, 1995; Fong et al., 1995; Vajkoczy et al., 1999). Although ECs are found at embryonic and extraembryonic sites, the resulting vessels are abnormally organized, apparently as a result of an overproliferation of ECs (Fong et al., 1995; Kearney et al., 2002), which suggests that this receptor negatively regulates or restrains angiogenesis. A third VEGFR, VEGFR3/Flt-4, is essential for lymphatic development, and it is activated by binding to VEGF-C. VEGFR3/Flt-4–deficient embryos also show defects in the formation of the circulatory system (Dumont et al., 1998).

VEGF-A plays an important role also during early postnatal life (Gerber et al., 1999). The partial inhibition of VEGF-A achieved by Cre-loxP–mediated gene targeting results in increased mortality, stunted body growth, and impaired organ development. More recently, a critical role for this factor has also been demonstrated during adult neovascularization (Grunewald et al., 2006). Together, these data account for the major position of the VEGF signaling system in vascular formation.

2. Angiopoietin/Tie Signaling

After the discovery of VEGF-A, a second family of growth factors important for EC survival and vascular remodeling was identified, with members of this family being called the *angiopoietins* (Davis et al., 1996; reviewed by Gale and Yancopoulos, 1999). The angiopoietins have been shown to have important functions during angiogenesis. Like the VEGFRs, the specificity of angiopoietins for vascular endothelium results from the restricted distribution of their tyrosine kinase receptors Tie1 and Tie2/Tek on ECs (Dumont et al., 1995; Sato et al., 1995). Angiopoietin 1 (Ang1) seems to be important for the stabilization of vessel walls by promoting interactions between vascular ECs and surrounding pericytes and smooth muscle cells. Consistent with a constitutive stabilizing role, Ang1 is widely expressed in adult normal tissues (Suri et al., 1996). In murine embryos that are deficient in Ang1, early stages of VEGF-dependent vascular development appear to occur rather normally. However, the remodeling and stabilization of the primitive vascular plexus is severely perturbed, and this leads to embryonic lethality. A similar phenotype (although it was more evident in the brain capillary plexus) was reported for murine embryos lacking Tie2 receptor (Sato et al., 1995). Alternatively, the transgenic overexpression of Ang1 leads to striking hypervascularization by promoting vascular maturation and inhibiting normal vascular pruning (Suri et al., 1998). Together, these results suggest a critical role for the Ang1/Tie2 system in the normal remodeling, maturation, and stabilization of the developing vasculature.

In sharp contrast with Ang1, angiopoietin 2 (Ang2) also binds to the Tie2 receptor, but it is unable to activate the Tie2 receptor, and thus it acts as a

natural antagonist for the Ang1/Tie2 interaction. The transgenic overexpression of Ang2 results in a lethal phenotype that is reminiscent of that seen in Ang1 or Tie2 knockout mice (Maisonpierre et al., 1997). Ang2 is highly expressed at sites of vascular remodeling, and it is hypothesized to destabilize mature vessels, thus rendering them more amenable to remodeling, regression, or additional angiogenic growth, depending on other signals that the vessels are receiving (most notably VEGF; Yancopoulos et al., 2000). Ang2 knockout mice have a complex phenotype (Gale et al., 2002) that includes some vascular defects, which supports a role for Ang2 in postnatal angiogenesis and/or vascular remodeling but which prominently includes malformations of the lymphatic vasculature. Large and small lymphatic vessels are generally able to be formed, but they have defects in their overall organization. The mice appear to be normal at birth, but soon after the start of feeding they develop severe defects as a result of lymphatic dysfunction, and they die around postnatal day 14. To help further clarify the role of Ang2 in blood and lymphatic vessel development, Gale et al. (2002) generated mice in which the Ang2 gene was replaced by Ang1. These mice showed an almost complete rescue of the lymphatic defects, but the postnatal remodeling defects of the retinal blood vasculature persisted. One interpretation of this phenotype is that Ang2 normally acts as a Tie2 agonist in the lymphatic vasculature (because Ang1 can rescue the defect), but it acts as a Tie2 antagonist in the retinal vasculature (because Ang1 cannot rescue the defect).

Like Tie2, the closely related Tie1 receptor is primarily expressed in vascular ECs (Sato et al., 1993). However, until recently, ligands for this receptor had not been identified, and it remained an orphan receptor. Vascular development proceeds normally in Tie1-deficient mice up to approximately embryonic day13.0, but shortly thereafter they begin to show signs of edema, local hemorrhage, and rupturing of microvessels, and they die between embryonic days 13.5 and 18.5 (Puri et al., 1995; Sato et al., 1995). Although previous studies failed to demonstrate the binding of angiopoietins to Tie1, it has recently been demonstrated that Ang1 and Ang4 may function as activating ligands for this receptor (Saharinen et al., 2005) and that its activation is promoted by the formation of heterodimeric complexes with Tie2 (Marron et al., 2000; Saharinen et al., 2005; Tsiamis et al., 2002). The mechanisms underlying the effects of this binding are not yet known, and it still remains unclear whether Tie1 can function as an independent receptor. The generation of mice with conditional null alleles or the development of more effective and specific inhibitors may reveal the precise roles of Tie signaling and of the different ligands and receptors during later development and in mature vessels.

II. ARTERIAL–VENOUS DIFFERENTIATION

The most fundamental dichotomy in the blood vascular system and one of the very first steps in the differentiation of endothelium is the specification of arterial and venous identity (reviewed by Eichmann et al., 2005; Lawson and Weinstein, 2002; Rossant and Hirashima, 2003; Torres-Vazquez et al., 2003). Although it has long been assumed that the specification of arterial versus venous endothelial fate was a late event in development defined primarily by anatomic sites and hemodynamic forces, evidence has recently begun to emerge that suggests that the identity of ECs is genetically determined before

the onset of circulation and before vessel assembly. Beginning with studies in the mouse showing that ephrinB2 and its receptor EphB4 are specifically expressed in arterial and venous endothelium, respectively (Wang et al., 1998), a large number of studies have now highlighted the signaling pathways involved in the differentiation of arteries and veins.

A variety of evidence indicates that arterial–venous identity is acquired early during vasculogenesis, well before the onset of blood flow. In murine (Wang et al., 1998), zebrafish (Lawson et al., 2001), and avian (Herzog et al., 2005) embryos, the differential expression of ephrinB2 and EphB4 (Lawson et al., 2001; Wang et al., 1998), NP-1 and NP-2 (Herzog et al., 2001), and other markers in arteries and veins precedes the initiation of blood flow (Figure 33.3). The fluorescent labeling of single angioblasts during early somitogenesis stages in the zebrafish showed that angioblasts give rise to either arterial or venous vascular ECs but not to mixed clones, thus further supporting the idea of early specification of arterial–venous identity (Zhong et al., 2001). Other evidence suggests, however, that there is considerably plasticity in the early vasculature with respect to arterial–venous identity. Grafting experiments in avian embryos indicate that EC fate remains plastic up to embryonic day 7, which is well after circulation is initiated. Ectopic grafts from embryonic quail arteries or veins can switch their cell fate after transplantation into host chick embryos (Moyon et al., 2001; Othman-Hassan et al., 2001), and time-lapse imaging experiments have shown that changes in circulatory flow patterns can regulate arterial–venous differentiation in the avian embryo yolk sac (le Noble et al., 2004). However, this plasticity is gradually lost later during development, perhaps as a result of association with VSMCs or other nonendothelial components of the vascular wall (Moyon et al., 2001). It seems likely that the overall vascular structure

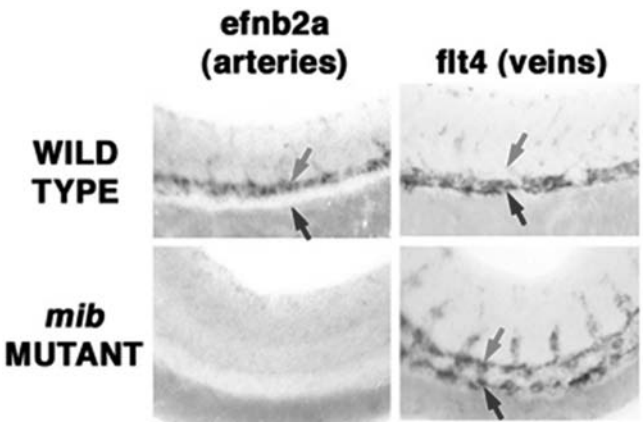

FIGURE 33.3 A reduction in Notch signaling in zebrafish embryos perturbs arterial–venous identity. *In situ* hybridization of the trunk dorsal aorta *(red arrows)* and cardinal vein *(blue arrows)* in 25-somite–stage Notch-deficient *mindbomb* (mib^{ta52b}) mutant and wild-type sibling zebrafish embryos. In wild-type animals, *ephrinB2a (efnb2a)* expression is apparent in the dorsal aorta but not the cardinal vein *(upper left)*. However, in notch-deficient mib^{ta52b} mutant embryos, *efnb2a* expression is absent *(lower left)*. By contrast, *flt4* expression is restricted to the cardinal vein in wild-type animals by the 25-somite stage *(upper right)*, whereas, in mib^{ta52b} mutant embryos, *flt4* expression persists within both the cardinal vein and the dorsal aorta *(lower right)*. All panels show lateral views of the mid trunk, dorsal up, anterior to the left. (Figure modified from Lawson et al., 2001. See color insert.)

during embryogenesis is defined by both the hemodynamics of circulatory flow and by the initial genetically programmed, intrinsic determination of arterial versus venous fate.

Recent work has shown a molecular cascade of events involved in the establishment of arterial–venous identity. We review some of these molecular players and their roles below.

A. Eph–Ephrin Signaling

The Eph-receptor tyrosine kinases constitute the largest known family of growth factor receptors, and they are activated by the equally numerous membrane-bound ephrins as their ligands (Adams et al., 1999; see Chapter 22). Although initially characterized in the nervous system, key roles for ephrinB and the EphB receptor in vascular development have been suggested in recent studies. In an initial knockout study in which the ephrinB2 locus was targeted with a *tau–lacZ* gene (Wang et al., 1998), the presence of this reporter was exclusively detected in the arteries even before the establishment of circulation, whereas in situ hybridization experiments on the same embryos showed the specific expression of its receptor, EphB4, in the venous endothelium. As noted previously, this was the first evidence for the genetic predetermination of arterial and venous fate. When the EphB4 locus was inactivated, vascular defects similar to those in mice lacking ephrinB2 were observed (Gerety et al., 1999). Animals carrying homozygous knock-in mutations of either the ligand or receptor display the proper differential expression of knocked-in transgenes and the normal initial formation of the major intraembryonic arterial and venous trunk vessels, which implies that this signaling pathway is not required for the first steps of arterial–venous specification. In both cases, however, later defects in the remodeling of the primary vascular plexus as well as in the maintenance of artery–vein separation were observed that led to death around embryonic day 9.5 (Adams et al., 1999; Gerety et al., 1999; Wang et al., 1998). Although ephrinB2 is also expressed in VSMCs, an endothelial-specific knockout of this gene displayed a very similar phenotype to the conventional ephrinB2 null mutant mice, thus demonstrating that the gene is critically required in ECs, at least for its earliest vascular functions (Gerety and Anderson, 2002). Interestingly, the complementary expression of ephrinB2 and EphB4 in arteries and veins is also present in adults, which suggests an important role for the reciprocal expression of these genes not only during development but also for the continued maintenance of proper arterial–venous differentiation in adults (Gale et al., 2001)

Molecularly, the ephrinB–EphB system can function bidirectionally. As for most other receptor tyrosine kinases, ligand binding induces "forward" signaling in EphB4, mainly through phosphotyrosine-mediated pathways. However, ephrins can also signal into their host cell (referred to as *reverse signaling*) via their cytoplasmic tail (reviewed by Kullander and Klein, 2002). The important role of ephrinB2 reverse signaling in angiogenesis was confirmed by recent studies of mice carrying a deletion of the cytoplasmic tail of ephrinB2 (Adams et al., 2001). In these mutant embryos, although the migration of neural crest cells (induced by Eph forward signaling) is normal, the remodeling of the vasculature is severely affected, and this suggests a critical role for ephrinB2 reverse signaling in this process. However, other researchers found that mice that were homozygous for novel knock-in alleles

of the ephrinB2 cytoplasmic tail targeting either the PDZ interaction site (ephrinB2 V/V) or the conserved tyrosine residues (ephrinB25F/5F) survived the initial requirement of ephrinB2 in embryonic vascular remodeling (Makinen et al., 2005). However, the ephrinB2 V/V mice exhibited major lymphatic defects, which suggests that there is an additional critical role for ephrinB2 reverse signaling via the PDZ interaction site in the postnatal remodeling of the lymphatic system.

In sum, although the expression of ephrinB2 in arteries and EphB4 in veins is required for normal vascular development, the fact that mice that lack ephrinB2 continue to express a *LacZ* transgene inserted at the ephrinB2 locus indicates that these genes are not required for the initial fate decision that distinguishes arterial and venous endothelial progenitors. This suggests that upstream factors may regulate the proper expression of arterial- and venous-specific genes as well as the determination of arterial–venous fate. Growing evidence suggests that the Notch signaling pathway plays a key role in this process.

B. Notch Signaling

The Notch signaling pathway is an evolutionarily conserved intercellular signaling mechanism that controls cell fate specification in a variety of tissues in nearly all animal species that have been investigated so far (Artavanis–Tsakonas et al., 1999; Chitnis, 1995). Several lines of evidence indicate an important role for Notch signaling during vascular development in vertebrates.

1. Members of the Notch Signaling Pathway Are Expressed in the Vasculature

Notch receptors and ligands are both present within the developing vasculature. Four different Notch receptors (Notch1 through Notch4) and five ligands (delta-like [Dll]1, Dll3, Dll4, Jagged-1 [Jag1], and Jag2), have been identified in mammals (Nye and Kopan, 1995). Notch1 (Del Amo et al., 1992; Reaume et al., 1992; Taichman et al., 2002), Notch2 (Del Amo et al., 1992; Zimrin et al., 1996), Notch3 (Villa et al., 2001), Notch4 (Krebs et al., 2000; Shirayoshi et al., 1997; Uyttendaele et al., 1996; Villa et al., 2001), and the Notch ligands Dll4, Jag1, and Jag2 are all expressed in vascular ECs during early embryogenesis in mice (Krebs et al., 2000; Shirayoshi et al., 1997; Villa et al., 2001). Members of the Notch family of receptors and ligands are also expressed in the vasculature in addition to various other tissues in other vertebrates such as chicken and zebrafish (Lawson et al., 2001; Vargesson et al., 1998; Zhong et al., 2000). Interestingly, the vascular expression of Notch receptors and ligands in the vasculature of different species has nearly always been reported to be restricted to arterial but not venous ECs (Lawson et al., 2001; Shutter et al., 2000; Villa et al., 2001), which suggests a role for this pathway during arterial differentiation.

2. Vascular Defects in Animals Deficient for Notch Receptors and Ligands

Genetic analysis in mice and humans has revealed various types of vascular defects associated with Notch pathway mutants. The dominant genetic disorder cerebral autosomal–dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is caused by mutations in the human Notch3 gene (reviewed by Kalimo et al., 2002). In patients with CADASIL,

vascular lesions occur throughout the arterial tree, including in the arteries and arterioles within muscle, skin, and peripheral nerves (Fryxell et al., 2001; Ruchoux et al., 1994; Schroder et al., 1995). Transgenic mice overexpressing murine Notch3 also exhibit vascular CADASIL–like phenotypes (Ruchoux et al., 2003). Alagille syndrome (AGS) is a genetic disorder caused by a mutation in the Notch receptor Jag1 (Xue et al., 1999). AGS is a major form of chronic liver disease in childhood, with severe morbidity and a mortality rate of 10% to 20% of affected individuals. Cardiac defects are also seen in more than 95% of patients with AGS (Gridley, 2003), and noncardiac vascular defects such as stenosis, aneurysm, and hemorrhage are also frequent in these patients, accounting for a third or more of their mortality (Emerick et al., 2005; Kamath et al., 2004).

Further evidence supporting the important role of the Notch signaling pathway in the formation and/or maintenance of the vasculature has come from *in vivo* studies carried out in mice, rats, and zebrafish. Notch1 and Notch1/Notch4 mutant mouse embryos display severe defects in angiogenic vascular remodeling, which lead to death from vascular defects and hemorrhaging at around embryonic day 10.5 (Krebs et al., 2000; Swiatek et al., 1994; reviewed by Rossant and Howard, 2002). By contrast, the expression of an activated form of Notch4 specifically in the embryonic endothelium leads to disorganized vascular development and a reduction of the number of small vessels, thus resulting in embryonic lethality at embryonic day 10.5 (Uyttendaele et al., 2001). These results indicate that either a loss or an excess activation of Notch receptors in the vasculature causes defects in blood vessel morphogenesis.

In similar fashion, mice lacking Notch ligands Jag1 and Dll1 die early during gestation as a result of severe defects in vascular remodeling (Barrantes et al., 1999; Xue et al., 1999). In addition, mutations in Dll4 (a Notch ligand expressed specifically in developing arterial ECs) lead to the defective development of the dorsal aorta and cardinal veins, the formation of arterial–venous shunts, and the downregulation of arterial markers and the upregulation of venous markers in the dorsal aorta (Duarte et al., 2004; Gale et al., 2004).

3. The Notch Signaling Pathway is Required for Arterial–Venous Cell Fate Determination

The artery-restricted pattern of expression of all identified vascular Notch signaling components suggested a specific role in arterial–venous cell fate determination, but it was not until recently that this role was confirmed by functional studies in zebrafish and mice. Zebrafish embryos deficient in Notch signaling as a result of a mutation in the *mindbomb (mib)* gene (Jiang et al., 1996) or of microinjection with a dominant–negative form of the transcription repressor Suppressor of hairless (*Su(H);* the common downstream effector of Notch signaling; Wettstein et al., 1997) display a loss of arterial markers such as ephrinB2a and Notch5 (Lawson et al., 2001; Zhong et al., 2001) that is accompanied by the ectopic expression of normally vein–restricted markers in the arteries (see Figure 33.3). Conversely, the activation of the Notch pathway by the either ubiquitous or endothelial-specific expression of Notch–intracellular domain induces the ectopic expression of artery markers in veins (Lawson et al., 2002). These data support the idea that arterial fate is specified and maintained by the Notch pathway via repression of the venous fate.

The *gridlock (grl)* gene (Zhong et al., 2001) has been reported to be a target of the Notch pathway in the zebrafish vasculature. The *grl* gene encodes a basic helix–loop–helix protein that belongs to the Hairy and Enhancer of split family of transcriptional repressors (Nakagawa et al., 1999). The vascular expression of *grl* is restricted to the aorta, and zebrafish embryos with mutations in the *grl* gene fail to establish trunk circulation as a result of the incomplete formation of the aorta (Stainier et al., 1995; Zhong et al., 2000). A number of studies have reported different results regarding the potential role of *grl* in arterial differentiation downstream of Notch. In one report, the injection of *grl* mRNA into wild-type zebrafish embryos repressed the expression of the venous markers *flt4* and *EphB4* (as did the injection of activated Notch5) and enhanced the expression of the arterial gene ephrinB2 (Zhong et al., 2001). However, in another study, *grl* was shown to be normally expressed in the dorsal aorta of embryos that lacked Notch activity, despite the ectopic expression of other artery–vein molecular markers and clear effects on vascular morphology (Lawson et al., 2001). These observations indicate that *grl* might not be the functional repressor of *flt4 in vivo*, and suggest the existence of other Hairy-related transcription factors that might mediate the repression of *flt4* downstream of the Notch pathway. The mammalian ortholog of *grl,* Hey2, is expressed in the developing cardiovascular system, and it has been shown to be a direct target of Notch signaling *in vitro* (Nakagawa et al., 2000). Although knockout of the Hey2 gene does not seem to affect artery/vein fate (Donovan et al., 2002; Fischer et al., 2002; Sakata et al., 2002), the double mutation of Hey2 and Hes1 (another Hairy-related transcription factor) produces a loss of arterial markers and vascular shunts, although dorsal aorta formation and morphology are also severely affected (Fisher and Caudy, 1998), which is not the case in Notch–deficient zebrafish.

Thus, the activation of Notch signaling seems to be a conserved requirement for the specification of arterialcell fate in vertebrates, but additional research will be required to clarify the potential role of different downstream response genes.

C. VEGF Signaling as a Regulator of Arterial–Venous Cell Fate

In addition to its general role in establishing and maintaining ECs (described previously), recent work demonstrated a more specific role for the VEGF system in the promotion of arterial endothelial differentiation upstream of Notch signaling (Lawson et al., 2002; Mukouyama et al., 2002; Stalmans et al., 2002). In cultured murine embryonic ECs, the addition of the VEGF-A isoforms $VEGF^{120}$ and $VEGF^{164}$ induce ephrinB2 expression up to 50%, whereas the addition of other growth factors, such as nerve growth factor and brain-derived neurotrophic factor, can only promote ephrinB2-positive cells to about 10% (Mukouyama et al., 2002). Recent studies have shown that postnatal mice that express only the $VEGF^{188}$ isoform (but not the predominant $VEGF^{164}$ or $VEGF^{120}$ isoforms) have reduced numbers of ephrinB2–positive arterioles in their retinas, whereas the number of venules and capillaries is unaffected (Stalmans et al., 2002). Conversely, mice that carry a $VEGF^{164}$ transgene have increased numbers of ephrinB2-positive capillaries and a concomitant decrease in EphB4-positive blood vessels in the heart (Visconti et al., 2002). Taken together, these results suggest that VEGF is sufficient to promote

arterial endothelial differentiation *in vivo* and *in vitro*, and it seems to do so independently of its ability to induce proliferation or the survival of all ECs.

The studies in mice are consistent with those in the zebrafish in demonstrating that VEGF is necessary and sufficient for arterial differentiation. A reduction of VEGF-A levels in zebrafish embryos by antisense morpholino injection prevents the expression of artery-specific markers such as ephrinB2, and it blocks the formation of arteries, whereas veins are largely unaffected. These defects can be rescued by the activation of Notch signaling (Lawson et al., 2002). However, VEGF is unable to rescue the arterial defects seen in embryos with reduced Notch activity. These results, together with additional experiments performed in zebrafish (Lawson et al., 2002) indicate that VEGF acts upstream of Notch signaling during arterial differentiation. Although analogous *in vivo* experiments to demonstrate VEGF functions upstream of Notch have not been performed in mammals, exogenous VEGF can induce the expression of Notch1 and Delta4 in human ECs *in vitro* (Liu et al., 2003).

The mechanisms underlying the artery-specific effects of VEGF–A remain unclear, because the major receptors (VEGFR-1 and VEGFR-2) are expressed on all ECs (reviewed by Ferrara et al., 2003; Klagsbrun and Eichmann, 2005). The NPs, which are VEGF coreceptors, are differentially expressed on arteries and veins. NP1 is preferentially expressed in arteries, whereas NP2 expression is restricted to the venous/lymphatic endothelium (Herzog et al., 2001). However, arterial–venous restriction precedes differential NP expression, and experiments carried out in both zebrafish and mice (Lawson et al., 2002; Mukouyama et al., 2002) support the idea that differential NP expression probably reinforces an arterial–venous decision that has already been initiated. Receptor output could also be regulated by the type of ligand available in the local environment and by genetic interactions among the different VEGF receptors contributing to blood vessel diversity during development (Covassin et al., 2006).

D. The Role of Sonic Hedgehog in Arterial–Venous Differentiation

Hedgehogs are a class of 19-kDa proteins that interact with heparin on the cell surface through an N-terminal basic domain and that are tethered to the surface through cholesterol and fatty acyl modification. Hedgehog signaling is crucial throughout development; Sonic hedgehog (Shh) is important for the determination of cell types in structures that lie adjacent to the notochord, and increasing evidence suggests a role for this signaling molecule in angiogenesis (reviewed by Lawson and Weinstein, 2002). Studies in zebrafish have shown that Shh acts upstream of VEGF to induce arterial endothelial differentiation (Lawson et al., 2002). The expression of VEGF is upregulated by Shh in both mouse (Pola et al., 2001) and zebrafish (Lawson et al., 2002) embryos, whereas its expression is lost in the somites and hypochord of zebrafish lacking Shh function, which results in the formation of a single midline axial blood vessel that expresses venous markers only (Lawson et al., 2002). In mice, Shh administered to aged animals induces new vessel growth in ischemic hind limbs, but Shh has no effect on EC migration or proliferation *in vitro*, although it does induce the expression of proangiogenic VEGF and angiopoietins-1 and -2 from interstitial mesenchymal cells (Pola et al., 2001). The two studies described suggest that Shh plays an indirect role in angiogenesis as an activator of other downstream angiogenic factors (Lawson et al., 2002; Pola et al., 2001), but other recent work suggests that Hedgehog

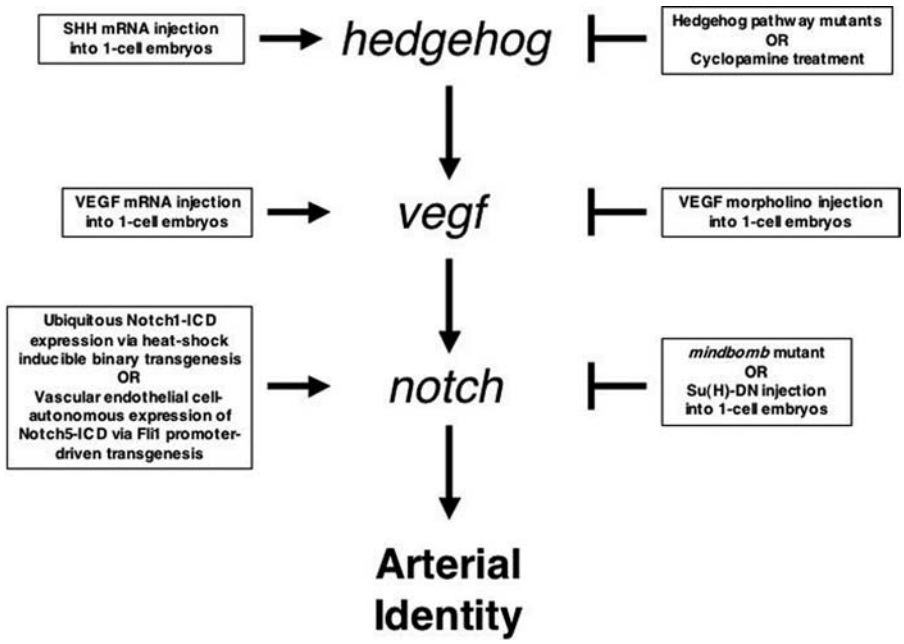
SHH mRNA injection into 1-cell embryos
hedgehog
Hedgehog pathway mutants OR Cyclopamine treatment
VEGF mRNA injection into 1-cell embryos
vegf
VEGF morpholino injection into 1-cell embryos
Ubiquitous Notch1-ICD expression via heat-shock inducible binary transgenesis OR Vascular endothelial cell-autonomous expression of Notch5-ICD via Flt1 promoter-driven transgenesis
notch
mindbomb mutant OR Su(H)-DN injection into 1-cell embryos
Arterial Identity

FIGURE 33.4 **A molecular pathway for arterial–venous fate determination.** Studies in the zebrafish have shown that vascular endothelial growth factor acts downstream of Sonic hedgehog and upstream of the Notch pathway to determine arterial cell fate. A variety of different methods were used to either increase *(left side)* or decrease *(right side)* the levels and/or activities of each of these signaling pathways, as shown. Loss of Notch, vascular endothelial growth factor, or Sonic hedgehog signaling results in the loss of arterial identity, whereas the exogenous activation or overexpression of these factors causes the ectopic expression of arterial markers. "Molecular epistasis" experiments were performed by combining different methods to assemble these components into an ordered pathway. (For further information about the zebrafish studies used to derive this pathway, see Lawson et al., 2001 and 2002).

signals may also be received by ECs directly to promote their proper morphogenesis into tubular vessels (Vokes et al., 2004).

Together, all of the studies described show that a molecular pathway consisting of the sequential activation of Hedgehog, VEGF, and Notch signaling regulates arterial differentiation in developing zebrafish (Figure 33.4) and probably also in mammals.

III. EMERGENCE OF THE LYMPHATIC SYSTEM

In addition to the blood vascular system, vertebrates possess a completely separate and parallel network of endothelial vessels called the *lymphatic vascular system*. Unlike the blood circulatory system, the lymphatic system is a blind-ended system of vessels that protect and maintain the fluid environment of the body by filtering and draining away lymphatic fluid. Lymphatic fluid is a clear, colorless fluid that contains water, dissolved molecules, and a few blood cells. The lymphatic system is not closed, and it has no single, central pump. The mammalian and avian lymphatic systems begin with innumerable blind-ended, thin-walled capillaries and larger vessels that drain lymphatic fluid from the extracellular spaces of all organs and tissues into larger

collecting tubes (Oliver, 2004). These vessels are lined with a continuous single layer of overlapping ECs that form loose intercellular junctions, which makes them highly permeable to large macromolecules, pathogens, and migrating cells. Larger lymph vessels have one-way, semilunar valves, and the lymph moves slowly and under low pressure as a result of the action of surrounding skeletal muscles, which help to squeeze fluid through them. This fluid is transported to progressively larger lymphatic vessels that culminate in the right lymphatic duct (for lymph from the right upper body) and the thoracic duct (for the rest of the body). These ducts drain into the blood circulatory system at the right and left subclavian vein.

Under normal conditions, the lymphatic vascular system is necessary for the return of extravasated interstitial fluid and macromolecules to the blood circulation, for immune defense, and for the uptake of dietary fats. It has an important role during embryonic development, and the growth and proliferation of lymphatic vessels is an essential feature of tissue repair and inflammation in most organs (Leu et al., 2000). Impaired functioning of lymphatic vessels can result in the formation of lymphoedema (Witte et al., 2001), whereas tumor-associated lymphangiogenesis may contribute to the spread of cancer cells from solid tumors. Thus far, it is unclear how tumor cells enter the lymphatic system; however, using lymphatic-specific molecular markers, many studies have shown that tumor cells activate peritumoral and intratumoral lymphangiogenesis (Mandriota et al., 2001; Skobe et al., 2001; Stacker et al., 2001).

In contrast with the extensive molecular and functional characterization of blood vascular endothelium, comparatively little is known about the mechanisms that control the formation, differentiation, and function of lymphatic vessels. The lack of specific markers has made it difficult to elucidate the mechanisms that underlie the development of the lymphatic system, and its origin has remained controversial. The most widely accepted view of early lymphatic development was described by Sabin (1902). On the basis of ink injection experiments, she postulated that the two primitive jugular lymph sacs originated from ECs that bud from large veins early during development. The peripheral lymphatic vessels subsequently form by centrifugal sprouting from these primary lymph sacs. More recent studies have provided support for this model, and they have suggested molecular players that might be important for different stages of lymphatic EC (LEC) emergence and specification (Figure 33.5).

Several studies with mice deficient in the homeobox transcription factor Prox-1 (Wigle and Oliver, 1999) or VEGF-C/VEGFR3 signaling (Karkkainen et al., 2004; Makinen et al., 2001) support this model. Prox-1, although broadly expressed during embryonic development, has been identified as a specific marker of a subpopulation of ECs that give rise to the lymphatic system (Oliver et al., 1993; Wigle and Oliver, 1999). As early as embryonic day 10.5, Prox-1–positive cells are detected in the wall of the cardinal vein. As development proceeds, these Prox-1–positive cells appear to bud from the cardinal vein to give rise to the lymphatic jugular sacs. Interestingly, the inactivation of Prox-1 in mice leads to a complete arrest of lymphatic system development (Wigle and Oliver, 1999). By contrast, vasculogenesis and angiogenesis are unaffected, which demonstrates that Prox–1 activity is specifically required for the normal development of the lymphatic system. These findings indicate that Prox–1 is a "master gene" in the program, specifying LEC fate.

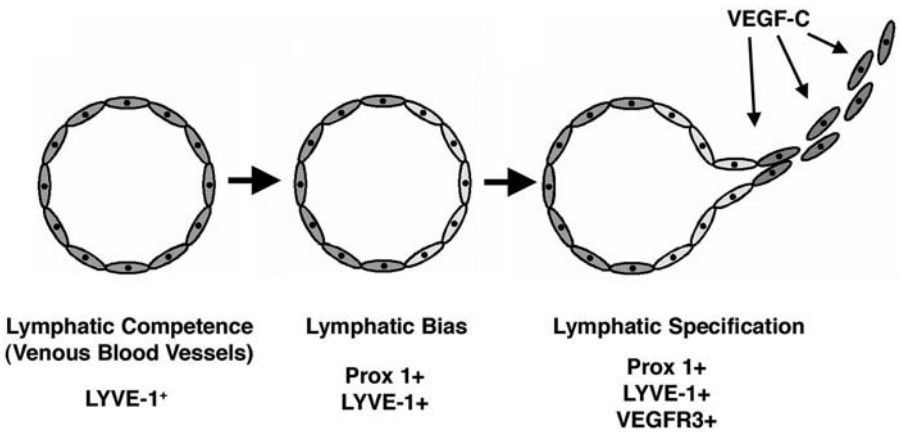

FIGURE 33.5 **A proposed model for lymphatic emergence from venous endothelium (modified from Oliver, 2004).** Lymphatic endothelium emerges from "competent" venous endothelium expressing the marker LYVE-1. Polarized expression of Prox-1 on a subset of venous endothelial cells "biases" these cells toward producing lymphatic endothelium and induces or allows for the continued maintenance of the expression of a number of different lymphatic endothelial cell genes, including vascular endothelial growth factor receptor 3, the receptor for the lymphangiogenic factor vascular endothelial growth factor C. (See Oliver [2004] for a more comprehensive discussion of this model.)

The signals that determine which ECs in the cardinal vein will activate Prox–1 and become lymphatic are unknown (Oliver and Detmar, 2002).

The migration of the LECs toward the lymph sacs was shown to be critically dependent on the presence of the growth factor VEGF-C. In mice that are deficient for this growth factor, Prox-1–expressing LECs are formed, but they fail to migrate toward the lymph sacs and subsequently die around embryonic day 17 as a result of the formation of massive lymphedema, because they do not develop any lymphatic vessels (Karkkainen et al., 2004). VEGF-C specifically binds to its high-affinity tyrosine kinase receptor VEGFR-3 (reviewed by Jussila and Alitalo, 2002). During mouse embryogenesis, the pattern of expression of VEGFR-3 (Flt-4) also coincides with Sabin's model of lymphatic development. VEGFR-3 is first expressed in a subset of blood vascular ECs, and it subsequently becomes restricted to LECs (Kaipainen et al., 1995; Oh et al., 1997)

Recent studies have demonstrated that signaling via VEGFR-3 is sufficient to induce lymphangiogenesis in transgenic mice (Jeltsch et al., 1997; Makinen et al., 2001). Moreover, the expression of a dominant–negative VEGFR-3 in the skin of transgenic mice blocks lymphangiogenesis and induces the regression of already formed lymphatic vessels, which demonstrates that VEGFR-3 signaling is required not only for the initial formation but also for the maintenance of the lymphatic vasculature (Makinen et al., 2001). However, consistent with the earlier expression of VEGFR-3 in the blood vasculature is the fact that VEGFR-3–deficient mice show defective blood vessel development at early embryonic stages, and the embryos die on embryonic day 9.5 (Dumont, 1998; Dumont et al., 1998). Thus, it seems that VEGFR-3 has an essential function in the remodeling of the primary capillary vasculature before the formation of the lymphatic vessels. VEGF-C also binds to the Neuropilin-2 receptor, which is specifically expressed by veins and

which subsequently becomes restricted to lymphatic vessels (Karkkainen et al., 2001; Yuan et al., 2002). Mice deficient for NRP-2 show selective defects in the formation of lymphatic vessels (Yuan et al., 2002).

An alternative model for the emergence of the lymphatic formation was proposed by Huntington and McClure (1910), who suggested that mesenchymal lymphangioblast-like cells are the source of lymphatic vessels and that lymphatics arise in the mesenchyme independent of the veins and then subsequently establish venous connections. This model has been supported by work performed in avian embryos by quail/chick grafting experiments (Schneider et al., 1999; Wilting et al., 2001, 2006) and in Xenopus (Ny et al., 2005), in which both transdifferentiated venous cells and lymphangioblasts have been reported to contribute to newly formed lymph vessels.

The debate surrounding LEC origins remains unresolved in large part because of the lack of an effective model organism that allows one to easily observe lymphatic cells *in vivo* and to perform defined genetic and experimental manipulation of the lymphatic system. Recently, however, the presence of a well-defined lymphatic system that shares characteristics of lymphatic vessels found in higher vertebrates was reported in the zebrafish (Yaniv et al., 2006). Using live imaging of transgenic zebrafish, researchers were able to trace the origins of LECs from their region of origin and through their incorporation into the thoracic duct (the main lymphatic vessel described in vertebrates), thus providing the first direct *in vivo* evidence for a venous origin for primitive lymphatic vessels, as proposed by Sabin a century ago. Further direct *in vivo* imaging studies in the zebrafish should similarly allow one to determine whether later-forming and/or peripheral lymphatics arise predominantly through the further proliferation of these initial LECs or via recruitment from mesenchyme as proposed for birds and frogs.

IV. PATTERNING OF THE DEVELOPING VASCULATURE

The gross vascular anatomy is characterized by a reproducible pattern of blood vessels. At least for the major vessels (e.g., the aorta), characteristic features such as lumen size, branching angles, and curvature along the vascular tree are quite reproducible. There are also designated sites for secondary sprouts (e.g., intersomitic vessels, main vessels penetrating different organs), whereas microvessels and capillaries formed by intussusceptive angiogenesis are mostly nonstereotyped. The control of branch patterning includes both attractive and repulsive guidance signals, and it is regulated by both positive and negative regulators.

The cellular and molecular mechanisms that govern blood vessel assembly at appropriate sites in the organism are poorly understood, yet understanding this regulation is critical to the ability to design therapeutics around vessel production *in vivo*. In the next few sections, we review recent advances in the understanding of how vascular patterning is established during embryonic development.

A. Assembly of the Primary Axial Vessels

The formation of the main axial vessels of the trunk—the dorsal aorta and the cardinal vein—occurs by vasculogenesis, which is the local aggregation

of angioblast progenitors arising in the mesoderm. Angioblasts specified in the lateral mesoderm migrate to the midline to form vascular cords that subsequently undergo morphogenesis into the lumenized vascular tubes of the axial vessels (the dorsal aorta and the cardinal vein). Evidence suggests that midline cues are important for the assembly of these initial vasculogenic vessels.

Studies in the zebrafish have shown that the notochord is required for dorsal aorta formation. Zebrafish embryos with mutations in the *floating head* (encodes *Xnot,* a homeobox gene; Schulte-Merker et al., 1994) or *no tail* (encodes *Brachyury;* Talbot et al., 1995) genes lack a differentiated notochord, and they also specifically lack a dorsal aorta, although they still form the cardinal vein (Fouquet et al., 1997; Sumoy et al., 1997). Wild-type notochord cells transplanted back into *floating head* mutants can locally rescue the assembly of aortic primordia (Fouquet et al., 1997), which suggests that notochord-derived signals are required for aortic specification. The *floating head* and *no tail* mutants also fail to form the hypochord, an endodermally derived thin strip of cells that lies immediately ventral to the notochord, just above the aorta in fish and amphibian embryos. Studies in Xenopus and zebrafish have shown that the hypochord expresses a soluble short isoform of VEGF that could potentially act as a medium to a long-range graded signal for the medial migration and assembly of the angioblasts that contribute to the immediately juxtaposed dorsal aorta (Cleaver and Krieg, 1998; Lawson et al., 2002; Liang et al., 2001). However, experiments designed to directly test this idea have not yet been performed, and studies in the zebrafish suggest that VEGF expressed by the adjacent somites in response to notochord-derived Hedgehog signals may be more critical than hypochord-derived VEGF for dorsal aorta assembly (Lawson et al., 2002).

The loss of VEGF activity causes a loss of dorsal aorta formation in mice, although it is not clear what tissues provide midline vascular patterning signals. Avians and mice lack a hypochord, but embryonic structures such as the somites or the primitive endoderm ventral to the aorta express VEGF and could be mediating arterial angioblast migration and aorta assembly. The role of the notochord in dorsal aorta formation in avians and mice is also not clear. Mouse *Brachyury* mutants lack the posterior notochord, but they still form a posterior dorsal aorta (Hogan and Bautch, 2004); however, this does not rule out the role of the notochord in the anterior region, where the *smoothened* phenotype is most severe (Vokes et al., 2004). In avians and mice, the dorsal aortae are initially present as a pair of vessels on either side of the midline rather than a single midline vessel, as they are in fish and amphibians. Studies using quail/chick chimeras in which axial structures were removed showed that the notochord acts as a midline barrier to impede avian angioblasts from crossing the axial midline (Klessinger and Christ, 1996), and a subsequent report showed that this was the result of notochord-expressed bone morphogenetic protein antagonists (Reese et al., 2004). Other recent studies using mouse/avian chimeras have shown that the neural tube is the source of a positive patterning signal, and they have identified this signal once again as VEGF-A (Ambler et al., 2001, 2003; Hogan and Bautch, 2004).

In addition to VEGF, *Hedgehog* signaling also seems to be important for the assembly of the dorsal aorta in zebrafish and mice (Ingham et al., 2000; Lawson et al., 2002; Vokes et al., 2004). As noted previously, notochord-derived

Hedgehog signaling has been shown to be important in the pathway leading to arterial differentiation in zebrafish because it induces VEGF expression in the somites. However, unlike deficiencies in Notch signaling, the loss of Hedgehog signaling leads to a complete loss of the dorsal aorta and not simply to its misspecification as vein, which suggests that Hedgehog signaling plays a role in dorsal aorta assembly in addition to its activity upstream of arterial differentiation (Lawson et al., 2002). Recent studies in Xenopus and mice confirm this, showing that Hedgehog signaling is important for the morphogenesis of a vascular tube (Vokes et al., 2004).

B. The Role of Guidance Factors in Developmental Angiogenesis

After the assembly of embryonic primary vasculogenic vessels such as the dorsal aorta and the cardinal vein, further remodeling and ramification of the vasculature are achieved by developmental angiogenesis, as described previously. Angiogenesis in adults is in most cases clearly directed and guided by local tissue requirements for oxygen and nutrients, but, during early development, it follows highly stereotypic and evolutionarily well-conserved patterns of vascular network assembly that are reminiscent of the way in which the initial assembly of the nervous system and axon tracts follows a conserved and stereotypic program of assembly (see Chapter 24). Indeed, the two systems display remarkable anatomic parallels, and vessels and nerves frequently course adjacent to one another. This has led to the idea that the assembly of the vasculature might depend on similar (or even the same) attractive and repulsive guidance cues from surrounding tissues that help to direct growing vessels along specific pathways (Weinstein, 2005). A variety of recent studies have begun to show that many of the molecular pathways used for the guidance of migrating axons are also employed in the direction of the stereotypic pathways followed by developing blood vessels.

The formation of the trunk intersegmental vessels provides a good example of a stereotypic and conserved process of vascular network assembly (Figure 33.6, A and B). Intersegmental vessels are conserved features of the trunk vasculature of all vertebrates. They form bilaterally on opposite sides of the trunk along each intersomitic boundary (vertical myoseptum). Studies in zebrafish have shown that these vessels grow and elongate in a choreographed and reproducible fashion (Isogai et al., 2003). Initially, primary vascular sprouts emerge from the dorsal aorta bilaterally at each intersomitic boundary, and they grow dorsally around the notochord and neural tube. As they reach the dorsal–lateral surface of the neural tube, they branch rostrally and caudally, fusing with adjacent intersegmental sprouts to form a pair of continuous vessels along the dorsal trunk called the *dorsal longitudinal anastomotic vessels*. The formation of the primary sprout-derived vascular lattice is followed by a wave of secondary vascular sprouts that emerge from the posterior cardinal vein. About half of these connect with the base of primary segments, which then become intersegmental veins. The rest of the primary segments, which remain connected only to the dorsal aorta, become intersegmental arteries. Recent studies have shown that well-known neuronal guidance factors play important roles in the guidance and patterning of these and other developing vessels, although in many cases the details of their vascular activities remain unclear.

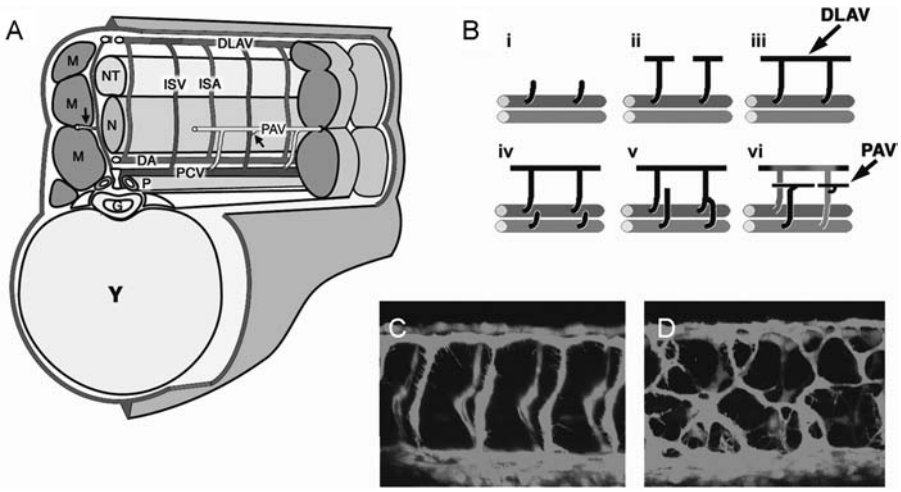

FIGURE 33.6 **Trunk vascular network assembly and its guidance. A,** The anatomy of the zebrafish trunk and its blood vessels by approximately three days postfertilization. At this stage, there is active flow through the dorsal aorta *(DA)*, the posterior cardinal vein *(PCV)*, and most intersegmental arteries *(ISA)* and intersegmental veins *(ISV)*. The intersegmental arteries and veins are linked together dorsally via paired dorsal longitudinal anastomotic vessels *(DLAV)*. All of these vessels are shown relative to adjacent tissues and structures in the mid trunk, including the gut *(G)*, the myotomes *(M)*, the notochord *(N)*, the neural tube *(NT)*, the left pronephric duct *(P)*, and the yolk mass *(Y)*. In addition to the functioning vessels noted here, parachordal vessels *(PAV)* run longitudinally to either side of the notochord, along the horizontal myoseptum. At 3 days postfertilization, the parachordal vessels do not yet carry flow. Anterior is to the left and above the plane of the page, and dorsal is up. **B,** Schematic diagram illustrating the steps that lead to the assembly of the trunk angiogenic vascular network. For clarity, the diagram shows the vessels on only one side of the trunk. **B.i,** Primary sprouts emerge bilaterally exclusively from the dorsal aorta *(red)*. **B.ii,** Primary sprouts grow dorsally, branching cranially and caudally at the level of the dorsal–lateral roof of the neural tube. **B.iii,** Branches interconnect on either side of the trunk to form two dorsal longitudinal anastomotic vessels *(DLAV)*. **B.iv,** Secondary sprouts begin to emerge exclusively from the posterior cardinal vein *(blue)*. **B.v,** Some secondary sprouts connect to the base of primary segments, whereas others do not. **B.vi,** Primary segments with patent connections to secondary segments become intersegmental veins *(blue)*, whereas primary segments that remain connected only to the dorsal aorta become intersegmental arteries *(red)*. Most of the secondary sprouts that do not connect to primary segments serve instead as ventral roots for the parachordal vessels *(PAV)*. **C** and **D,** Blood vessels in the mid trunk of control morpholino (**C**) or plexinD1 morpholino (**D**) injected 48 hour postfertilization into *fli1*–EGFP transgenic embryos. In control morpholino-injected animals, intersegmental vessels extend along the boundaries between somites, avoiding the semaphorin-rich central regions (**C**). In animals deficient in plexinD1, intersegmental vessels sprout, branch, and grow without regard for somitic boundaries (**D**). Anterior is to the left and dorsal is up in all panels. (Panels A and B are modified from Isogai et al., 2003. Panels C and D are modified from Torres–Vazquez et al., 2004. See these references for further details. See color insert.)

1. Semaphorin Signaling

Some of the most conclusive evidence for neuronal guidance factors playing important roles in guiding and patterning the developing vasculature has come from studies of semaphorin signaling and blood vessels in zebrafish and mice. Semaphorins are a large family of cell-associated and secreted proteins that signal through multimeric receptors (Bagri and Tessier-Lavigne, 2002). Membrane-associated semaphorins bind directly to plexin receptors, whereas secreted semaphorins bind to NP-plexin receptor complexes. Recent

work has shown that semaphorin–plexin signaling regulates the guidance and patterning of the vasculature in a manner similar to that of the repulsive guidance roles of semaphorins in the nervous system. ECs express various NP and plexin receptors, including the endothelial-specific receptor plexinD1 (Basile et al., 2004; Gitler et al., 2004; Miao et al., 1999; Soker et al., 1998; Torres-Vazquez et al., 2004). In zebrafish, type 3 semaphorins are expressed in the center of each somite, and semaphorin-plexinD1 signaling mediates the repulsive guidance of growing intersegmental vessels to restrict their paths to semaphorin-free corridors along the intersomitic boundaries (Torres-Vazquez et al., 2004). The loss of function of either the plexinD1 receptor or the trunk semaphorins in zebrafish causes the mispatterning of intersegmental vessels, which sprout at irregular positions and grow and branch aberrantly throughout the trunk instead of maintaining paths along the intersomitic boundaries (see Figure 33.6, *C* and *D*).

In mice, the targeted inactivation of plexinD1 also causes the mispatterning of intersegmental vessels as well as increased vascularization of the somites, which normally exclude vessels (Gitler et al., 2004; Gu et al., 2005). In this case, the primary ligand is apparently Sema3E, which is normally expressed in a region of the somites that is adjacent to the intersegmental vessels (Gu et al., 2005), although the murine plexinD1 receptor is capable of responding to Sema3A in an NP-dependent manner (Gitler et al., 2004). However, a mouse mutant that is deficient in semaphorin binding to both NP1 and NP2 forms intersegmental vessels normally, which suggests that Sema3E binds plexinD1 directly rather than via an NP receptor. Further studies will be needed to work out the precise ligand–receptor interactions for semaphorin signaling in the vasculature.

2. Slit–Robo Signaling

Several recent studies have implicated Slits and their receptors in angiogenesis. There are four slit receptors ("Roundabout" receptors or Robos) in mammals (Robos). Robo4, which is structurally divergent from the other Robos, shows highly endothelial-cell–specific expression both *in vitro* and during mouse embryogenesis (Park et al., 2003). In the adult, Robo4 is present at sites of both normal and pathologic active angiogenesis, including that of tumor vessels (Huminiecki et al., 2002). The role of Slit–Robo signaling in vascular guidance remains controversial. One study showed that Robo4 binds Slit0002 and that it is able to inhibit the migration of Robo4-expressing cells *in vitro* (Park et al., 2003), but other studies either failed to detect such binding (Suchting et al., 2005) or demonstrated a promigratory effect of Slit2 on ECs (Wang et al., 2003). Substantial vascular defects have not been reported in either Robo4 or Slit ligand knockout mice, although this could be explained by redundancy in the expression of both ligands and receptors (Long et al., 2004). However, defects in intersegmental vessel formation have been reported in zebrafish after the morpholino-mediated knockdown of Robo4 (Bedell et al., 2005). Again, further studies will be required to determine thenature of the *in vivo* role of Slit–Robo signaling during vascular development.

3. Netrin Signaling

Another important set of guidance cues during nervous system patterning is provided by the netrins, which are a family of highly conserved laminin-

related secreted proteins (Hedgecock et al., 1990; Ishii et al., 1992; Serafini et al., 1994). Netrins can mediate the attraction of neurons by activating Deleted in colorectal cancer (DCC) receptor family members that are expressed on axons (Fazeli et al., 1997; Serafini et al., 1996), including DCC and neogenin (Chan et al., 1996; Keino-Masu et al., 1996). Conversely, netrin binding to members of the Uncoordinated-5 (UNC5) receptor family results in axon repulsion. Like Slits, the role of netrins in the guidance of the vasculature is still somewhat unclear. Several different studies carried out both *in vitro* and *in vivo* in mice and zebrafish have shown that netrin-1 is a proangiogenic factor for vascular ECs (Park et al., 2004; Wilson et al., 2006), but another study has shown that netrin-1 can act as a repellent in vessel-guidance via the UNC5B receptor (Lu et al., 2004). In the zebrafish, netrin-1a is expressed along the horizontal myoseptum that divides the dorsal and ventral halves of the somites. One report suggests that the loss of netrin-1a prevents the formation of the parachordal vessels that normally run through the horizontal myoseptum, which suggests that netrin-1a provides a positive cue for the growth of these vessels (Wilson et al., 2006). However, another report suggests that the loss of netrin-1a in the zebrafish promotes increased vessel branching and growth along the somites (Lu et al., 2004). The differing conclusions of all of these studies may at least partly reflect the biology of netrins, which are capable of both repulsive and attractive signaling, depending on the cellular and environmental context. Further studies will be required to clarify the differences between these and other results regarding the activity of netrins and their receptors in the vasculature.

There is clearly much to be done before our understanding of how guidance factors regulate vascular patterning begins to approach the depth of current understanding of how these factors regulate neural patterning. The expression of many of these factors adjacent to or in opposition with developing vessel tracts suggests that they play important roles in establishing the anatomic form of vascular networks (Figure 33.7), but conclusive *in vivo* evidence is, in most cases, still lacking. It is likely that, as is seen in the nervous system, extensively redundant guidance factors for the vasculature coordinate their activities in a complex spatial and temporal interaction to shape the stereotypic pattern of early blood vessels. The strong parallels uncovered between these two systems already imply that studies carried out in the nervous system that are aimed at dissecting this interaction will continue to have relevance to the vascular system (and, perhaps, vice versa).

V. CONCLUDING REMARKS

The differentiation of ECs and the formation of the vascular system are of central importance during embryonic vascular development. Research carried out over the past years has shed light on the different steps involved in the formation of the vascular system. The emergence of endothelial progenitors, their coalescence into the primary vascular system (vasculogenesis) and the further remodeling and elaboration of the initial plexus (angiogenesis), the differentiation of vessels into arteries and veins, and the formation of the lymphatic system have been extensively studied. Genetic evidence has highlighted the roles of many molecules that affect vasculogenesis, angiogenesis, and lymphangiogenesis in a complex and tightly regulated manner. Many

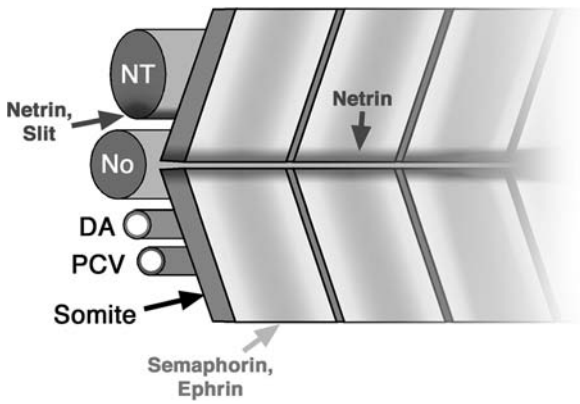

FIGURE 33.7 Members of various families of guidance factors are expressed in discrete patterns in the developing trunk. Netrins and Slits are expressed in the ventral neural tube. Netrin is expressed along the horizontal myoseptum, and semaphorins and ephrins are expressed within the somites. These signaling molecules are well positioned to provide potential repulsive or attractive guidance cues for blood vessels such as the intersegmental vessels (which run vertically along intersomitic boundaries), the parachordal vessels (which run longitudinally along the horizontal myosepta), and the vertebral arteries (which run longitudinally on either side of the ventral neural tube). *NT,* Neural tube; *No,* notochord; *DA,* dorsal aorta; *PCV,* posterior cardinal vein (Image from Weinstein, 2005.)

of the signaling pathways implicated in vascular development are reactivated during disease states of angiogenesis or vessel regression, thus making a full understanding of the complexities of these pathways important for identifying new targets for therapeutic intervention during pathologic situations such as severe tissue ischemia, coronary heart disease, and tumor-promoted angiogenesis.

A particularly interesting aspect of recent research is the heterogeneity displayed by ECs. To date, it is clear that, although all vessels share the same endothelial basis, each cell type has acquired unique characteristics that are vital to cardiovascular system function. To what extent is the formation of different types of vessels defined solely by intrinsic programs or influenced by local cues? How does the intimate association with their cognate organs influence ECs to adopt functional specialties such as the blood–brain barrier and the fenestrated endothelium in the kidney glomeruli? The answers to these questions are of vital importance when aiming to refine therapeutic applications to specific subsets of the vasculature.

Much of the past decade of research on vascular development has focused on the central role played by VEGF and its receptors. Although there is still much more to uncover about VEGF signaling, a challenge for the coming decade will be to incorporate our understanding of the role of this pathway into a larger framework of multiple and sometimes highly specific regulators. Genetic, molecular, and cell biologic tools are now available for the study of vessel formation in a diverse array of model systems, thus creating a wide and useful array of tools for vascular research. Because many of our insights into the mechanisms underlying the formation of the vascular system have come from developmental studies, it seems likely that further work involving the early stages of vascular formation will continue to shed light on the activities of these pathways during normal and pathologic adult neovascularization.

SUMMARY

- Primitive blood vessels in vertebrate embryos form by a process called *vasculogenesis,* during which mesodermal cells differentiate into endothelial precursor cells called *angioblasts*. These angioblasts then differentiate in situ into ECs, and they coalesce to form the earliest vessels. The subsequent growth and remodeling of the vasculature occur by angiogenesis, during which new blood vessels form from preexisting vessels by endothelial sprouting and splitting.
- The VEGF signaling pathway has been shown to be important for the migration, proliferation, maintenance, and survival of ECs and to be critical during both vasculogenesis and angiogenesis. In addition to its important function during embryonic development, VEGF is thought to play a major role in tumor-induced neovascularization. Angiopoietin–Tie signaling is important for the maturation and stabilization of the nascent vascular network.
- One of the most fundamental steps in the differentiation of the vasculature is the specification of arterial and venous endothelium. Recent work has highlighted the role of Notch, VEGF, and Hedgehog signaling in the specification of arterial identity. Classically, differences between arteries and veins were attributed to physiologic factors such as the direction and pressure of blood flow. However, recent work has shown that molecular distinctions between arterial and venous endothelium appear before the onset of blood flow, and it has highlighted the roles of genetic pathways including Notch, VEGF, and Hedgehog signaling in specifying arterial identity.
- Vertebrates possess a second, blind-ended vascular system, the lymphatic system, that is responsible for clearing and draining fluids and macromolecules that leak from blood vessels into the interstitial spaces of tissues and organs. Evidence suggests that the first LECs emerge by transdifferentiation from the ECs of primitive veins. The transcription factor Prox1 is a critical regulator of LEC specification, whereas the VEGF family members VEGF-C and VEGF-D are important for LEC migration and lymphangiogenesis.
- During early development, newly formed vessels follow a highly stereotypic and evolutionarily well-conserved pattern of network assembly that is reminiscent of that followed by the nervous system and axon tracts. A variety of recent studies have shown that many well-known molecular pathways used for the guidance of migrating axons play an important role in the guidance and patterning of developing blood vessels.

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GLOSSARY

Angioblast
Mesodermal-derived endothelial precursors that have certain characteristics of endothelial cells but that have not yet assembled into functional vessels.

Angiogenesis
The formation of new blood vessels from preexisting vessels by endothelial sprouting and splitting. This process of remodeling of the primary capillary network leads to the formation of mature arteries and veins.

Lymphatic system
A blind-ended system of vessels that protect and maintain the fluid environment of the body by filtering and draining away lymphatic fluid. Under normal conditions, the lymphatic vascular system is necessary for the return of extravasated interstitial fluid and macromolecules to the blood circulation, for immune defense, and for the uptake of dietary fats.

Vasculogenesis
The process of the formation of primitive vessels in vertebrate embryos during which mesodermal cells differentiate into endothelial precursor cells called *angioblasts*. These angioblasts then differentiate in situ into endothelial cells and coalesce to form the earliest vessels.

Vascular endothelial growth factor
The first growth factor described to be a specific mitogen for endothelial cells. It is important for the migration, proliferation, maintenance, and survival of endothelial cells, and it is critical during both vasculogenesis and angiogenesis. It also plays an important role in arterial differentiation.

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RECOMMENDED RESOURCES

Coultas L, Chawengsaksophak K, Rossant J: Endothelial cells and VEGF in vascular development, *Nature* 438:937–945, 2005.
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34 BLOOD INDUCTION AND EMBRYONIC FORMATION

XIAOYING BAI and LEONARD I. ZON

Howard Hughes Medical Institute, Children's Hospital of Boston, Boston, MA

INTRODUCTION

Each day, our body produces billions of new white blood cells, red blood cells, and platelets to replace the blood cells lost during the process of cell turnover. These mature blood cells are generated by a small population of stem cells called *hematopoietic stem cells* (HSCs) that reside in the bone marrow. HSCs are defined by their capacity of self-renew, and their multilineage differentiation can give rise to all blood cells in our body, including erythrocytes, granulocytes (basophils, eosinophils, and neutrophils), lymphocytes (B cells, T cells, and natural killer cells), monocytes/macrophages, and platelets (Figure 34.1). When transplanted into a host that has been lethally irradiated to remove endogenous HSCs, the donor-derived HSCs can reconstitute all blood lineages throughout life span (Fleischman et al., 1982; Harrison et al., 1988; Spangrude et al., 1988; Jordan and Lemischka, 1990; Chaddah et al., 1996; Osawa et al., 1996).

The emergence of HSCs and the blood system can be traced back to the early stages of embryogenesis. Pioneering studies addressing the formation of the blood system were performed in chick embryos because of the easy accessibility of embryos for observation and manipulation. Later, this work was extended into mammalian models such as the mouse. More recently, Xenopus and zebrafish models have been extensively used to decipher the molecular pathways involved in hematopoiesis. These studies have revealed a remarkably conserved developmental program of hematopoiesis. In addition, *in vitro* studies using hematopoietic progenitor cell culture and embryonic stem cell (ESC)-derived embryonic bodies greatly contribute to our understanding of the hematopoietic hierarchy. In this chapter, we will review the general process of hematopoiesis in these vertebrate model organisms and highlight the important molecules that regulate blood development.

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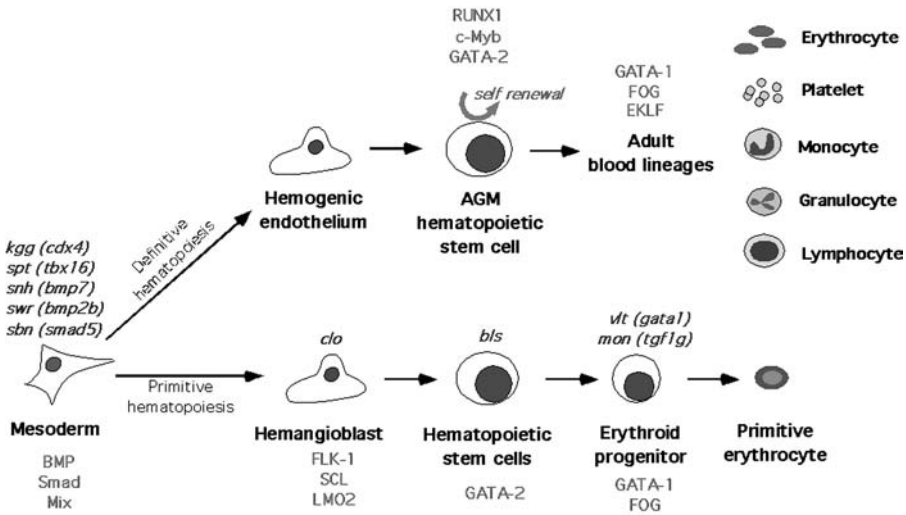

FIGURE 34.1 **The general process of hematopoiesis in the vertebrate.** Important regulatory factors are shown. The zebrafish mutants are in italics, with the mutant gene indicated in parentheses.

I. ORIGIN OF BLOOD CELLS DURING EMBRYOGENESIS

A. Induction of Hematopoiesis in the Early Embryo

In vertebrates, blood cells derive from the ventral mesoderm (see Chapter 13). During gastrulation, the mesoderm is induced and subsequently patterned to adopt a dorsal or ventral fate. Studies in Xenopus and zebrafish have uncovered mesoderm-inducing factors that include members of the transforming growth factor β (TGF-β) family and the fibroblast growth factors (Munoz-Sanjuan and H-Brivanlou, 2001). In addition, the T-box transcription factor *VegT* has also been found to be important for mesoderm formation (Zhang et al., 1998). The zebrafish mutant *spadetail,* which is caused by the loss of function of *tbx16* (the homologue of *VegT*), displays a severe defect in the mesodermal and endodermal derivatives in the trunk, including an absence of blood (Kimmel et al., 1989; Ho and Kane, 1990; Thompson et al., 1998).

Patterning of the mesoderm is regulated by antagonistic interactions between the ventralizing signals and the dorsalizing signals (Graff, 1997; Thomsen, 1997). Considerable evidence suggests that the ventralizing signals are mediated by members of the bone morphogenetic proteins (BMPs), a subgroup in the TGF-β superfamily. The overexpression of *BMP2, BMP4,* or *BMP7* results in the loss of dorsal derivatives (e.g., the muscle, the notochord), and it expands ventral mesoderm fates (e.g., the blood, the kidney; Dale et al., 1992b; Jones et al., 1992; Fainsod et al., 1994; Clement et al., 1995; Wang et al., 1997). Animal cap (ectodermal) explants in Xenopus revealed that the ectopic expression of *BMP4* induces the molecular markers of hematopoiesis, such as *Gata-2* and *Scl* (Maeno et al., 1996; Mead et al., 1998a). Conversely, blocking BMP pathways by the overexpression of a dominant-negative BMP receptor inhibits blood formation and causes an expansion of the dorsal derivatives (Graff et al., 1994; Maeno et al., 1994;

Suzuki et al., 1994). Furthermore, several zebrafish mutants with disruptions in the BMP pathway, such as *swirl* (*BMP2* mutant), *snailhouse* (*BMP7* mutant), and *somitobun* (mutant of *Smad5,* the downstream signal transducer of the BMP pathway), lack the ventral tissues (i.e., dorsalized mutants) and have defects in blood formation (see Figure 34.1; Mullins et al., 1996; Kishimoto et al., 1997; Nguyen et al., 1998; Hild et al., 1999; Dick et al., 2000; Schmid et al., 2000). Conversely, a defect in the *chordin* gene ventralizes the zebrafish embryo and expands blood formation, thus suggesting that *chordin* is a dorsalizing signal. Similar dorsalizing molecules are also identified in Xenopus, including *chordin, noggin,* and *follistatin* (Piccolo et al., 1996; Zimmerman et al., 1996; Iemura et al., 1998). The overexpression of these factors dorsalizes the ventral mesoderm by interacting with BMP signals to prevent receptor activation. Gene knockout studies in mice also support the role of the BMP pathways in mesoderm patterning and blood induction. Targeted disruption of mouse *BMP4, BMP2,* or *Alk3* (a BMP receptor) genes resulted in severe mesoderm deficiency, and, in certain genetic backgrounds, primitive hematopoiesis in these mutant embryos was severely disrupted (Winnier et al., 1995; Lawson et al., 1999). Recently, members of the Hedgehog family (which are secreted by the visceral endoderm in mouse embryos) have been found to appear to be capable of inducing blood formation in mouse embryo explant cultures, perhaps through the upregulation of *BMP4* (Dyer et al., 2001). In addition to its general effect on hematopoiesis through dorsal–ventral patterning, a recent study in zebrafish showed that *Alk8,* a BMP receptor, regulates the specification of the myeloid lineage during early embryogenesis, thus suggesting that BMP/TGF-β signaling may also regulate hematopoiesis in a lineage-specific pattern (Hogan et al., 2006).

The BMP pathways are possibly mediated by the *Mix* family of transcription factors, which belongs to the paired class of homeobox genes (Mead et al., 1998b). Seven distinct *Mix* factors have been isolated in *Xenopus*, including *Mix.1, Mix.2, Mix.3, Bix1, Bix2, Bix3,* and *Bix4* (Mead et al., 1996; Vize, 1996; Ecochard et al., 1998; Henry and Melton, 1998; Tada et al., 1998). *Mix* genes are transiently expressed in the future mesoderm and/or the endoderm during gastrulation, and they are induced by activin (another TGF-β family member) and BMP. An activin response element has been identified in the *Mix.2* promoter (Chen et al., 1997). The overexpression of *Mix.1* induces excessive blood formation and ventralizes the embryos in a way that is similar to that seen with the BMP overexpression phenotype (Dale et al., 1992a; Jones et al., 1992). The single *Mix* gene homologue in mouse, *Mixl1/mMix,* is expressed in the primitive streak of the gastrulating embryo, and it marks the cells that are destined to form mesoderm and endoderm (Pearce and Evans 1999; Robb et al., 2000). *Mixl1* null mice display numerous mesodermal and endodermal defects that result in embryonic lethality at day 8.5 postcoitum (dpc). Using *Mixl1* null ESCs, Elefanty et al. recently demonstrated that *Mixl1* is required for efficient hematopoiesis and *BMP4*-induced ventral mesoderm patterning (Ng et al., 2005). Conversely, the induction of *Mixl* in ESC-derived embryonic bodies results in the acceleration of mesoderm development and the expansion of hematopoietic progenitors (Willey et al., 2006). Taken together, these studies suggest that the *Mix* family may participate in the BMP signaling pathways in patterning the mesoderm toward a ventral and hematopoietic fate.

B. From Hemangioblast to Hematopoietic Stem Cells

When embryonic hematopoiesis initiates, hematopoietic and endothelial cells emerge simultaneously in close association with each other from the mesoderm. The developmental proximity between these two cell types has led to the hypothesis that they arise from a common progenitor called the *hemangioblast*. Numerous studies have suggested such a common origin for hematopoietic and endothelial lineages. For example, mice lacking *Flk-1,* which is an early endothelial marker, fail to generate both endothelial and hematopoietic cells (Shalaby et al., 1995). *Flk-1* null ESCs also fail to contribute to either vessels or blood cells in chimeric mice (Shalaby et al., 1995). Furthermore, single *Flk-1*$^{+}$ cells from avian embryos can develop into either hematopoietic or endothelial cells, depending on the presence of the vascular endothelial growth factor (Eichmann et al., 1997). Similarly, *Scl,* which is an early hematopoietic marker, is also required for the endothelial lineage, because *Scl* null ESCs fail to contribute to the formation of the vitelline vessels in the mouse yolk sac (YS; Visvader et al., 1998). In support of the mouse studies, a zebrafish mutant *Cloche* lacks both endothelial and hematopoietic cells, and *Scl* expression is greatly reduced (Stainier et al., 1995; Liao et al., 1997). The overexpression of *Scl* can partially rescue both blood and vascular defects, which suggests that the mutant gene functions upstream of *Scl,* perhaps at the hemangioblast level (Liao et al., 1998).

Using *in vitro* differentiated ESCs, Choi et al. (1998) have isolated a blast colony-forming cell (BL-CFC) population. BL-CFCs behave like hemangioblasts in that they express a number of genes that are common to both endothelial and hematopoietic lineages, and they have potential to form either lineage. Recently, Huber et al. (2004) have identified BL-CFCs in the posterior region of the primitive streak of gastrulating mouse embryo, thereby providing evidence for the existence of hemangioblasts *in vivo*. Most interestingly, adult HSCs isolated from human bone marrow and human cord blood have been shown to have vascularizing potential, thereby suggesting that hemangioblasts are also present during the postnatal stage (Pelosi et al., 2002).

C. Primitive Hematopoiesis

Hematopoiesis in vertebrate embryos is characterized by two successive waves occurring at anatomically distinct sites. Primitive hematopoiesis is transient, generating cells mainly in the erythroid lineage (see Figure 34.1), although macrophages and megakaryocytes are also found in primitive hematopoiesis. The second wave, which is called *definitive hematopoiesis,* lasts for the life of the organism and produces HSCs that are capable of giving rise to all blood lineages (see Figure 34.1).

In mouse embryos, the first hematopoietic cells appear extraembryonically, within the mesoderm-derived YS blood island (Figure 34.2; Palis et al., 1999; 2001). The expression of the hematopoietic genes *Scl* and Lim only domain 2 *(Lmo-2)* marks the initiation of YS hematopoiesis at 7 dpc (Palis et al., 2001). Subsequently, *Gata-1,* which is an erythroid-specific transcription factor, is detected in the YS (Pevny et al., 1995; Palis et al., 2001). Most of the primitive blood cells are red cells (erythrocytes). Different from the enucleated definitive red cells, primitive erythrocytes retain their nucleus, and they predominantly express the embryonic hemoglobins (ζ, βH1, and εy;

Steiner, 1973). Between 8.5 and 9 dpc, the primitive erythrocytes enter the circulation, and, by 9 dpc, the primitive erythropoietic potential of the YS has disappeared (Palis et al., 1999). Similar to mammals, avian primitive hematopoiesis initiates in the YS (see Figure 34.2). Differentiated primitive red blood cells are detected at day 1.5, and embryonic circulation starts by day 2 (Evans, 1997).

In Xenopus, the ventral blood island (VBI) is functionally equivalent to the mammalian and avian YS, which also develops from the ventral mesoderm (Mangia et al., 1970). By 24 hours postfertilization (hpf), *Scl, c-Myb,* and *Gata-1* can be detected in the developing VBI (see Figure 34.2; Turpen et al., 1997). Primitive erythrocytes start to express the hemoglobin at 40 hpf, and, by 50 hpf, the circulation is established (Mangia et al., 1970).

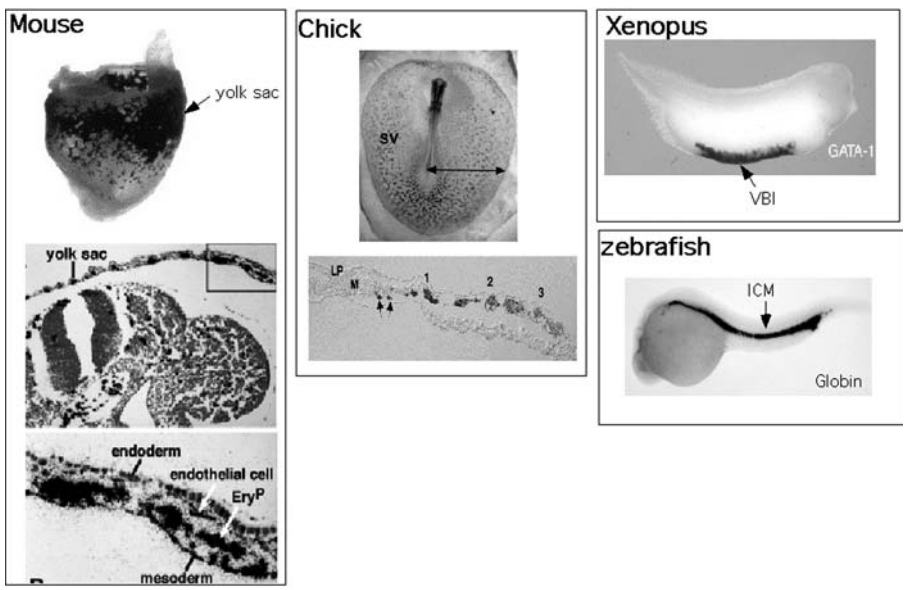

FIGURE 34.2 **The site of primitive hematopoiesis in different species. Mouse: Upper panel,** A mouse embryo (~8.5 dpc) from an ε-globin lacZ transgenic mouse line stained for the expression of β-galactosidase *(dark blue)* in primitive erythroid cells in the yolk sac. **Middle panel,** The in situ hybridization of an embryonic globin probe to a 10.5-dpc mouse embryo section showing the cellular composition of the yolk sac. The yolk sac is seen as a membrane surrounding the embryo, and it contains a series of blood islands. Two of these *(rectangle)* are shown at higher magnification in the lower panel. **Lower panel,** Visceral endoderm and mesoderm components are seen enveloping clusters of primitive erythroblasts *(Ery^P)* surrounded by endothelial cells. *Chick:* **Upper panel,** A 2-day-old (10 somite pairs) chick embryo showing the expression of the *Lmo-2* gene. The dotted pattern reveals the distribution of the blood islands, which will give rise to the first (primitive) generation of erythrocytes. *SV,* Sinus venosus. **Lower panel,** Cross-section at the level indicated previously showing the maturation steps of the blood islands *(BI)*. Arrows indicate the hemangioblasts that will give rise to the endothelial cell *(EC)* and the hematopoietic cell *(HC)*. **1,** This immature blood island is full of hematopoietic and endothelial cells that are already differentiated. **2,** The blood island has matured; several cells have been freed, creating a hole. **3,** The blood island is fully mature. The hematopoietic cells are free and detached from one another. *LP,* Lateral plate; **M,** mesoderm. *Xenopus:* In situ hybridization of the *Gata-1* probe to a swimming tadpole-stage embryo reveals the high expression of *Gata-1* in the ventral blood island region. *Zebrafish:* The *in situ* hybridization of an embryonic globin probe to a 24 hpf embryo reveals the intermediate cell mass region. (Mouse figures adapted from Baron, 2001, with permission. Chick figures adapted from Jaffredo et al., 2003, with permission. Xenopus figures adapted from Mead et al., 2001, with permission. See color insert.)

Unlike other vertebrate systems in which primitive hematopoiesis occurs extraembryonically, primitive hematopoiesis in zebrafish takes place in the embryo proper at the intermediate cell mass (ICM). Derived from the posterior intermediate mesoderm, the ICM precursors are first evident around the 2-somite stage by the expression of the hematopoietic marker *Scl* in bilateral stripes of cells flanking the paraxial mesoderm (Davidson et al., 2003). At the 4-somite stage, the expression of the erythroid-specific transcription factor *Gata-1* is detected in a subset of Scl^{+} cells (Davidson et al., 2003), thus indicating the erythropoietic commitment of the ICM precursors. Erythroid precursors then migrate toward the trunk midline to form the ICM, and, at 15 hpf, they begin expressing embryonic globins (see Figure 34.2; Al-Adhami and Kunz, 1977; Willett et al., 1999). Between 24 and 26 hpf, the heart starts beating, and the erythroblasts enters the circulation, where they subsequently mature into primitive erythrocytes (Willett et al., 1999).

D. Definitive Hematopoiesis and Hematopoietic Stem Cells

Primitive hematopoiesis is transient and subsequently replaced by the definitive wave of hematopoiesis, which generates HSCs that give rise to all adult blood lineages throughout the life span. Definitive HSCs colonize the fetal liver (FL) and later the bone marrow; however, their origin has been controversial during the past few decades. Using quail–chick chimeras in which the embryo of a quail was grafted onto the YS of a chick, Dieterlen-Lievre (1975) showed that the chick-derived YS cells only contribute to primitive but not definitive hematopoiesis in the quail embryo. Further inspection showed that definitive HSCs come from intraembryonic regions that are closely associated with the aorta (Dieterlen-Lievre and Martin, 1981). Similarly, in mouse embryos, definitive hematopoietic progenitors have been detected at 9 dpc in the aorta–mesonephros–gonads (AGM) region. At 10 dpc, AGM-derived cells have long-term repopulating potential in lethally irradiated adult recipients lacking the endogenous HSCs (Muller et al., 1994), thus demonstrating HSC activity in the AGM. Recently, the AGM origin of definitive HSCs was also demonstrated in zebrafish (Figure 34.3, C; Thompson et al., 1998). Morphologically, putative hematopoietic cells appear as clusters budding from the ventral wall of the dorsal aorta as well as from the endothelium of vitelline/umbilical arteries, and they express HSC markers such as *Flk-1, Scl,* and *Runx-1* (see Figure 34.3; Garcia-Porrero et al., 1995; Marshall et al., 1999; North et al., 1999; Tavian et al., 1999). In *Runx-1*–deficient mouse embryos, the formation of the intra-aortic clusters is disrupted, and definitive hematopoiesis is blocked (North et al., 1999). Therefore, a hypothesis of "hemogenic endothelium" (see Figure 34.1) has been proposed in which definitive HSCs are generated through an endothelial intermediate that has the potential to give rise to hematopoietic cells (Jaffredo et al., 1998).

In addition to the AGM region, definitive hematopoietic potential has been described in the mouse YS. Palis et al. (1999) discovered definitive erythroid progenitors expressing the adult globin in the YS at 8 dpc, before the onset of circulation. As circulation begins, these cells are found in the bloodstream and subsequently in the liver, which suggests that YS-derived erythroid cells can colonize the FL. YS cells taken at 9 to 10 dpc can provide long-term (up to 1 year) multilineage blood reconstitution for conditioned newborn recipients, but they cannot be engrafted into irradiated adult recipients (Yoder et al., 1997a;

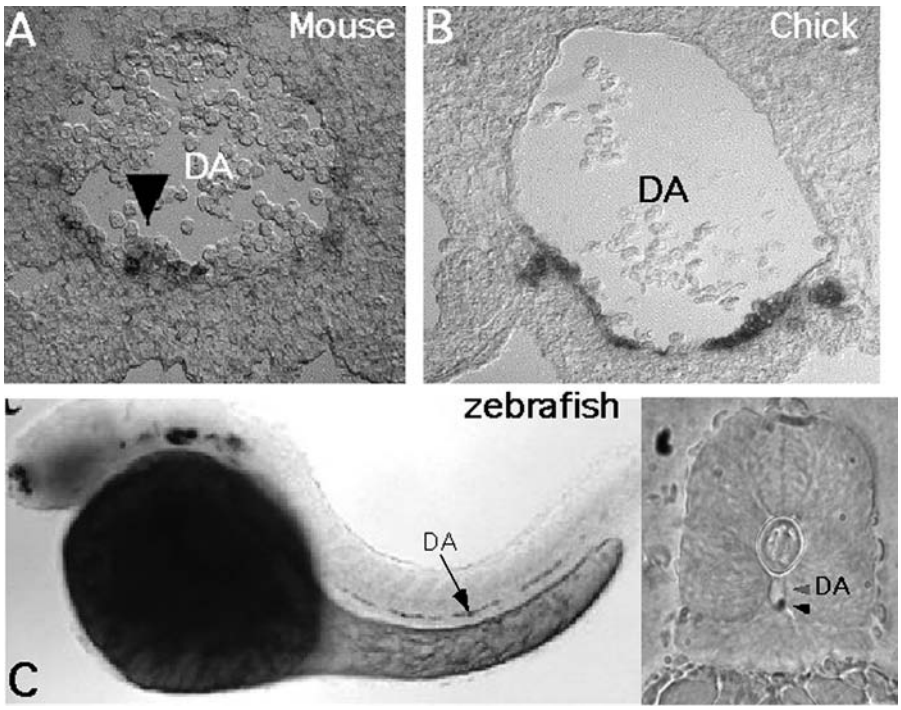

FIGURE 34.3 **The sites of definitive hematopoiesis in different species.** The *in situ* hybridization of the *Runx-1* probe in **A,** mouse, **B,** chick, and **C,** zebrafish embryos reveals *Runx-1* expression in the ventral endothelium of the dorsal aorta. In **C,** the cross section of the dorsal aorta is shown on the right, with the dorsal aorta *(red arrowhead)* and the *Runx-1* in situ signal *(blue arrowhead)* indicated. (Adapted from Jeffredo et al., 2005, with permission. See color insert.)

1997b); When they are cultured with AGM-derived stromal cells, however, YS cells isolated at 8 dpc provide long-term HSCs in adult recipients (Matsuoka et al., 2001), which suggests that the YS-derived cells have the potential to become definitive HSCs given the proper environmental cues.

Although the contribution of YS-derived cells to definitive hematopoiesis in mice is still under debate, the VBI in Xenopus has been demonstrated to give rise to both primitive and definitive hematopoiesis. As mentioned previously, primitive hematopoiesis occurs at the VBI in Xenopus. The second site of amphibian hematopoiesis, the dorsal–lateral plate, is analogous to the mammalian AGM (Kau and Turpen, 1983; Maeno et al., 1985). Both VBI– and dorsal–lateral-plate–derived cells are found to colonize the liver and the thymus, where they later give rise to all blood lineages in larvae and adults (Kau and Turpen, 1983; Smith et al., 1989; Bechtold et al., 1992; Chen and Turpen, 1995).

Recent studies have suggested that there may be new anatomic sites participating in HSC development during vertebrate embryogenesis. Caprioli et al. (1998; 2001) have discovered cells with hemangioblastic potential in the avian allantois. Similarly, hematopoietic stem cell activity has been described in the murine placenta, which is an equivalent structure to the avian allantois. Alvarez-Silva et al. (2003) found that the murine placenta contains a large number of hematopoietic progenitors that can differentiate into multilineages when they are cultured *in vitro*. Gekas et al. (2005) showed that HSCs with long-term reconstitution activity could be found in the placenta before their appearance in the bloodstream, which is

suggestive of the de novo hematopoietic potential of the placenta. Moreover, between 11.5 and 12.5 dpc, the placental HSC pool expands, and this results in more than 15 times the number of HSCs as compared with the number found in the AGM region(Gekas et al., 2005). This expansion of placental HSCs may result from the continuous generation of HSCs in the placenta or the homing of HSCs to the placenta from other sites. Further studies are required to verify the origin of placental HSCs.

E. Colonization of Hematopoietic Organs by Embryonic Precursors

After their birth in the AGM and/or the YS, HSCs migrate to the newly forming hematopoietic organs, where they reside in specific locations called *stem-cell niches,* which provide a microenvironment for HSC self-renewal and differentiation. In the stem cell niches, HSCs undergo extensive proliferation and differentiation, and they give rise to all blood lineages to support the growth of the organism.

The migration of HSCs from the AGM and/or the YS to the hematopoietic organs is suggested by the quantitative temporal measurement of HSC activity during mouse embryogenesis. In mouse embryos, HSC activity in the AGM and YS decrease after 11 dpc, and it becomes undetectable by 13 dpc (Muller et al., 1994; Sanchez et al., 1996). This decrease in HSC activity in the AGM and YS is accompanied by an exponential increase in HSC activity in the FL from 12 to 15 dpc and later the in bone marrow (Morrison et al., 1995). Although the mechanisms of HSC migration and colonization are largely unknown, several recent studies have suggested possible regulatory factors. Mice deficient in β1-integrin (an adhesion molecule) have hematopoietic progenitors that are present in the YS, the AGM, and the bloodstream; however, these progenitors are unable to properly seed the hematopoietic organs, and this results in a complete absence of fetal hematopoiesis (Potocnik et al., 2000). Chemokine–chemokine-receptor interactions have also been implicated in the migration of HSCs. Mice deficient in the chemokine stromal-cell–derived factor 1α *(SDF-1α)* or its receptor, *CXCR4,* fail to establish bone marrow hematopoiesis, although the FL hematopoiesis is normal (Zou et al., 1998; Godin et al., 1999; Ara et al., 2003), which suggests a critical role for *SDF-1α–CXCR4* interaction in HSC migration to the bone marrow>. Consistent with these results, Wright et al. recently demonstrated that FL HSCs migrate in response to *SDF-1α in vitro* (Wright et al., 2002). Moreover, the migratory response of FL HSCs is greatly enhanced in the presence of another chemokine, stem cell factor (*SCF;* Christensen et al., 2004), which has well-established roles in HSC maintenance, survival, and proliferation. The mutation of the *SCF*-encoding gene or its receptor *c-Kit* gene leads to profound hematopoietic defects (Russell, 1979). Consistent with a possible function in cell migration, *SCF* and *c-Kit* are expressed along the migration pathways of the germ cells, the melanocytes, the central nervous system, and the hematopoietic cells.

In mice, the FL predominates hematopoiesis from 12 dpc through birth, giving rise to all blood lineages to support the growing fetus (Delassus and Cumano, 1996; Mebius and Akashi, 2000; Mebius et al., 2001). At the end of fetal life, the spleen becomes a predominant erythropoietic organ, and it aids in the transition from FL to the bone marrow hematopoiesis (Sasaki and Matsumura, 1988; Godin et al., 1999). The bone marrow is the last

hematopoietic organ to develop in the fetus, but it is the primary niche for HSCs in the adult. The bone marrow HSC reservoir is largely established during postnatal life, when liver hematopoiesis ceases. Other vertebrates use different hematopoietic organs during the fetal and adult stages. For example, in Xenopus, the liver is the main hematopoietic organ in both the larval and adult stages (Chen and Turpen, 1995), whereas, in the avian system, the site of hematopoiesis shifts directly from the AGM to the bone marrow (Dieterlen-Lievre and Martin, 1981). In zebrafish, the kidney maintains the larval and adult hematopoiesis (Willett et al., 1999).

II. GENETIC APPROACHES TO THE STUDY OF THE TRANSCRIPTION FACTORS IN BLOOD DEVELOPMENT

The process of HSC generation, proliferation, and differentiation involves complex interactions of transcription factors that modulate the genetic switches along a particular developmental pathway. During the past few decades, many transcription factors have been identified as being essential for blood development by targeted gene disruption in the murine system. More recently, zebrafish has become a powerful model organism for the systematic genetic analysis of vertebrate development, particularly with regard to hematopoiesis. Extensive genetic screens in zebrafish have yielded at least 26 complementation groups of blood mutants (Ransom et al., 1996), thus providing a great source for the genetic dissection of vertebrate hematopoietic pathways.

A. Targeted Gene Disruption in Mice

The ability to disrupt specific genes by homologous recombination in murine ESCs has allowed investigators to address loss-of-function questions in a mammalian system (Capecchi, 1989). Targeted mutations in mice have identified several important transcription factors that act at distinct stages during hematopoiesis.

1. Transcription Factors Acting at the Hematopoietic–Stem-Cell/Progenitor Level

Flk-1 is a tyrosine kinase receptor for the vascular endothelial growth factor (VEGF). It is expressed in the extraembryonic mesoderm that is destined to give rise to both the vascular and hematopoietic components of the YS blood island of the mouse embryo (Yamaguchi et al., 1993). Expression is maintained in the endothelial cells as well as in the primitive hematopoietic progenitors. Mice that are homozygous for the disruption of *Flk-1* die between 8 and 9 dpc, because they lack both the vascular and the primitive blood progenitors (Shalaby et al., 1995). Furthermore, *Flk-1*–null ESCs fail to contribute to either the vessels or the blood cells in chimeric mouse embryos (Shalaby et al., 1995). These findings suggested that *Flk-1* plays essential functions in both blood and endothelial development, perhaps at the hemangioblast stage. However, another model suggests that *Flk-1* does not play an instructive role in hematopoiesis. Hidaka et al. (1999) found that, by altering the culture condition, *Flk*-deficient ESCs can give rise to hematopoietic lineages *in vitro* in differentiated embryonic bodies, thereby suggesting

that *Flk-1* regulates the migration of hemangioblasts to the proper environment that is permissive to hematopoiesis (Hidaka et al., 1999).

Scl/Tal-1, which was originally identified in a chromosomal translocation in T-cell acute lymphoblast leukemia, encodes a basic helix–loop–helix transcription factor (Hershfield et al., 1984; Begley et al., 1991). By heterodimerizing with E2A products (E12 and E47), *Scl* recognizes the E-box motifs (CANNTG; Hsu et al., 1994; Shivdasani and Orkin, 1996). It can also form complexes with *Gata, Ldb-1,* and *Lmo-2* to regulate the erythroid differentiation (Wadman et al., 1997). *Scl* expression is detected in hematopoietic, vascular, and neuronal tissues (Green et al., 1992; Drake et al., 1997). *Scl* null mice die around 8.5 dpc, and they completely lack blood cells; *Scl* null ESCs fail to contribute to any definitive hematopoietic lineage, thereby demonstrating the essential role of *Scl* in the formation of HSCs (Shivdasani et al., 1995; Porcher et al., 1996). Although *Scl* is essential for the genesis of HSCs, the conditional knockout of *Scl* in adult mice does not disturb the HSC function, which suggests that *Scl* is not continually required for the identity and function of HSCs (Mikkola et al., 2003). The function of *Scl* in endothelial lineage has also been suggested. Although *Scl*-deficient embryos have some vascular endothelial cells, *Scl* null ESCs fail to contribute to the formation of the vitelline vessels in chimera mouse embryos, which suggests that *Scl* is required for the late events in endothelial development (Visvader et al., 1998).

The *Lmo-2* gene is also involved in a chromosomal translocation in T-cell acute lymphoblast leukemia (Rabbitts, 1998). It encodes a Lim-domain protein that acts as a bridge between DNA-binding transcription factors such as *Scl* and *Gata-1* (Wadman et al., 1997). The disruption of *Lmo-2* in mice causes lethality at 9 dpc, with a complete lack of YS hematopoiesis (Warren et al., 1994). *Lmo-2* null ESCs do not contribute to any adult blood lineage or to the endothelial cells of large vessels (Yamada et al., 1998), thus demonstrating that *Lmo-2* has a similar function to *Scl* in hematopoiesis and angiogenesis.

Gata-2 belongs to the Gata family of transcription factors, which contain two homologous zinc-finger domains and which bind to the Gata consensus sequence (T/AGATAA/G; Crispino et al., 1999; Shimizu et al., 2001). It is highly expressed in the extraembryonic mesoderm, the immature blood progenitors, and the HSCs (Minegishi et al., 1999; 2003). *Gata-2* null mice have markedly reduced primitive and definitive hematopoiesis, and they die around 10 to 11 dpc (Tsai et al., 1994). Hematopoietic progenitors from *Gata-2* null ESCs proliferate poorly *in vitro,* and they undergo extensive apoptosis (Tsai and Orkin, 1997), which suggests that *Gata-2* is essential for the proliferation and survival of early progenitors. Furthermore, studies involving the use of *Gata-2* heterozygous mice revealed that the *Gata-2*$^{+/-}$ bone marrow has reduced numbers of HSCs and that the cells exhibit a higher frequency of cell death; this suggests that the dose of *Gata-2* is important for the maintenance of adult HSC homeostasis (Rodrigues et al., 2005).

Core binding factor (CBF) is a heterodimeric transcriptional factor that consists of a DNA-binding subunit *Runx-1* (also known as *AML1/CBFA2/PEBP2αB*) and a non–DNA-binding subunit CBF-β (Ogawa et al., 1993a; 1993b; Wang et al., 1993). *Runx-1* belongs to the *Runx* family of transcription factors, which bind to DNA through an evolutionarily conserved Runt domain. CBF-β associates with *Runx-1* and enhances its DNA-binding affinity. Mice that are deficient in either subunit lack all definitive blood lineages, but primitive hematopoiesis is not affected (Okuda et al., 1996; Sasaki

et al., 1996; Wang et al., 1996a; 1996b). Hematopoietic colony assays have revealed that the AGM cells from *Runx-1*$^{-/-}$ embryos cannot form any blood lineage, which suggests that CBF is required at the level of stem cells (Mukouyama et al., 2000). In support of this, *Runx-1* expression is found in the endothelial cells lining the ventral aspect of the dorsal aorta in the AGM region as well as in other intra-aortic sites from which hematopoietic clusters are thought to emerge (see Figure 34.3). In *Runx-1*–deficient mice, no hematopoietic clusters are generated, which suggests that CBF is required for the "budding" of definitive HSCs from the intra-aortic endothelium (North et al., 1999). In the adult, however, conditional knockout studies in mice have suggested that *Runx-1* is not required for the maintenance of HSCs in the adult bone marrow (Ichikawa et al., 2004).

c-Myb is the cellular homolog of the *v-Myb* oncogene. It is highly expressed in immature hematopoietic progenitors, but its expression decreases as they differentiate (Shivdasani and Orkin, 1996). Mice lacking *c-Myb* have normal primitive hematopoiesis but a marked loss of definitive progenitors in the FL, which results in death at 15 dpc (Mucenski et al., 1991). AGM cells from *c-Myb*$^{-/-}$ embryos do not generate hematopoietic cells *in vitro* (Mukouyama et al., 1999); this is indicative of there being an essential function of *c-Myb* during early definitive hematopoiesis.

2. Lineage-Specific Transcription Factors

Gata-1, which is the founding member of the Gata family of zinc-finger transcription factors, serves as a central regulator for erythroid gene transcription and development. The Gata motif has been found in virtually all characterized erythroid-specific genes (Evans et al., 1988). Disrupting *Gata-1* function in mice results in embryonic lethality at 11.5 dpc from fatal anemia caused by a block in erythroid differentiation at the proerythroblast stage accompanied by apoptosis (Pevny et al., 1991; 1995; Fujiwara et al., 1996), thus demonstrating its critical role in erythroid commitment and differentiation. In addition, the selective knockout of *Gata-1* expression in megakaryocytes blocks megakaryocyte differentiation (Shivdasani et al., 1997). Conversely, the forced expression of *Gata-1* in a myeloid progenitor cell line promotes megakaryocytic and erythroid differentiation (Visvader et al., 1992). Taken together, these studies establish an instructing role of *Gata-1* in megakaryo/erythroid lineage specification and differentiation.

Fog is a multitype zinc-finger protein that has been identified as a binding partner of *Gata-1* in yeast two-hybrid screens. It is coexpressed with *Gata-1* during hematopoietic development, and it cooperates in the mediation of erythroid and megakaryocytic differentiation. Mutant *Gata-1* that is unable to interact with *Fog* fails to support erythroid maturation (Crispino et al., 1999), which suggests a crucial role of *Gata-1*–*Fog-1* interaction during erythropoiesis. The targeted disruption of *Fog* in mice leads to blocked erythropoiesis (Tsang et al., 1998); the result is similar to the phenotype of *Gata-1*–knockout mice, thus further supporting *Fog*'s cooperative function with *Gata-1*.

Eklf is an erythroid Kruppel factor that belongs to the Kruppel zinc-finger protein family, which binds a CACC motif that has been found in many erythroid-specific genes, including adult β-globin (Miller and Bieker, 1993; Feng et al., 1994; Crossley et al., 1996). *Eklf* null mice die at the FL stage as a result of severe anemia and β-globin deficiency (Nuez et al., 1995). Primitive

erythropoiesis and embryonic globin expression are normal, and they demonstrate the pivotal role of *Eklf* in the activation of adult β-globin expression during the late stages of erythropoiesis (Perkins et al., 1995).

B. The Zebrafish Bloodless Mutants

Over the past 20 years, zebrafish has been proven to be a powerful model for large-scale genetic investigations in vertebrates. The unique advantages of zebrafish, such as external fertilization and embryogenesis, the large brood size, and the relatively short developmental period, greatly facilitate the forward genetic screens. The transparent zebrafish embryo is especially helpful for the detection and analysis of blood mutants. To date, at least 26 complementary groups with hematopoietic defects have been isolated, and they can be categorized into four phenotypic classes: bloodless, hypochromic, decreasing blood, and photosensitive mutants (Ransom et al., 1996). Here, we will focus on the bloodless class, in which no or few blood cells can be detected in the circulation that begins at 24 hpf.

1. *spadetail (spt)*

The *Spt* mutation is caused by a defect in the *tbx16* gene, which encodes a T-box transcription factor (Griffin et al., 1998). In *spt* embryos, the mesodermal cell migration is affected, and the paraxial–mesodermal cells are mislocated to the tail, which results in aberrant somite patterning (Kimmel et al., 1989). In addition, *spt* embryos have specific defects in primitive erythropoiesis in the trunk region, but they retain the normal development of macrophage and myeloid lineage in the head (Thompson et al., 1998; Amacher et al., 2002). Overexpression of *Scl* can rescue blood defects in *spt* embryos (Dooley et al., 2005), which suggests that *spt* acts upstream of *Scl*. Accordingly, the expression of early hematopoietic markers such as *Scl, Lmo-2,* and *Gata-2* and of the erythroid marker *Gata-1* is absent in the trunk hematopoietic precursors, but it is retained in the developing endothelial cells. Although the mesoderm-patterning defect in *spt* embryos may lead to the disruption in the blood specification, Rohde et al. (2004) used transplantation assays to find that *spt* function is required both cell-autonomously for hematopoiesis and non–cell autonomously for creating the proper environment for red cell development.

2. *kugelig (kgg)*

The *kgg* mutant is characterized by severe anemia, a shortened tail, and reduced yolk tube extension (Hammerschmidt et al., 1996). The defective gene is *cdx4,* which is a member of the caudal-related homeobox transcription factor family (Davidson et al., 2003). The *cdx* family in vertebrates has been implicated in the anterior–posterior patterning of the embryonic axis through the regulation of the *Hox* genes. Consistent with the role in *Hox* regulation, the expression pattern of at least nine *Hox* genes is altered in *kgg* mutants. The number of ICM precursors expressing *Scl* and *Gata-1* is also reduced, which indicates an early defect in hematopoiesis. This is not caused by a general posterior patterning defect resulting from perturbed *Hox* gene expression, because the adjacent pronephric tissue is normally patterned in *kgg* mutants. Instead, the *Hox* genes have been implicated to have an integral role in hematopoiesis, because the overexpression of *Hoxb7* and *Hoxa9* rescues the

blood defect without correcting the morphologic defects in *kgg* (Davidson et al., 2003). Recently, using muse ESC culture, Wang et al. (2005) found that ectopic *cdx4* expression promotes hematopoietic mesoderm specification, increases hematopoietic progenitor formation, and, together with *HoxB4*, enhances the multilineage hematopoietic engraftment of lethally irradiated adult mice. Taken together, these studies demonstrate the specific function of the cdx–*Hox* pathway in vertebrate blood development.

3. *cloche (clo)*

The *clo* mutants have virtually no blood and vascular cells, and they lack heart endocardium (Stainier et al., 1995). Gene expression analyses of HSC markers and angioblast markers including *Scl, Lmo-2, Gata-2, Runx-1, Fli-1,* and *Flk-1* have revealed a near complete absence of hematopoietic and endothelial lineages in *clo* embryos (Liao et al., 1998). The overexpression of *Scl* but not *BMP4* can at least partially rescue the *clo* defect, which suggests that *clo* acts downstream of *BMP4* but upstream of *Scl,* perhaps at the hemangioblast level. The mutant gene in *clo* has not yet been cloned as a result of the telomeric location of the gene (Liao et al., 2000). Uncovering the mutation responsible for *clo* is expected to provide insight into the molecular events that direct the commitment of mesoderm toward the blood and/or endothelial fates.

4. *bloodless (bls)*

The *bls* mutant is especially interesting in that the blood defect is restricted to primitive hematopoiesis. Mutant embryos are bloodless until 5 days postfertilization (dpf; Liao et al., 2002), after which the blood cells begin to repopulate the animal, thereby allowing the embryos to survive to adulthood. *Scl* and *Gata-1* expression are greatly reduced during primitive hematopoiesis in the ICM region, and rare specified hematopoietic progenitors undergo apoptosis (Liao et al., 2002). Definitive hematopoiesis is delayed in *bls* mutants. At 36 hpf, *c-Myb* expression in the AGM is weak as compared with that of the wild-type embryos; however, by 48 hpf, *c-Myb* expression is recovered in the *bls* mutants, which suggests the recovery of definitive hematopoiesis (J. Galloway, unpublished result). Similarly, the expression of *Rag1,* which is a marker of lymphocytes, is absent in the *bls* embryos at 4.5 dpf but recovered by 7.5 dpf. Although the defect gene is not yet cloned, it has been speculated that the *bls* gene may encode a secreted signal on the basis of its non–cell-autonomous defect (Liao et al., 2002).

5. *vlad tepes (vlt)*

The *vlt* mutant is caused by a nonsense mutation in the zebrafish *Gata-1* gene that results in a truncated protein that is unable to bind DNA or to mediate Gata-specific transactivation (Lyons et al., 2002). The mutant embryos have a severe reduction in erythroid progenitors, and this results in few or no blood cells at the onset of the circulation. Expression analyses reveal the normal expression of early hematopoietic markers such as *Scl* and *Lmo-2* but a great reduction or even the absence of a number of erythroid markers throughout development, thus demonstrating that the fundamental role of *Gata-1* in erythropoiesis is conserved in zebrafish. Recently, Galloway et al. (2005) showed that the loss of *Gata-1* function transforms the erythroid

precursors into myeloid cells, thereby demonstrating that *Gata-1* is required to determine erythroid versus myeloid fate during blood development.

6. *moonshine (mon)*

Mon mutants are characterized by severe anemia, increased apoptosis in ICM and the tail fin, and the enhanced proliferation of iridophores (Ransom et al., 1996). The expression of hematopoietic markers such as *Scl* and *Lmo-2* and of the erythroid marker *Gata-1* is initiated normally, which suggests that the erythroid precursors are formed in *mon* embryos. However, these precursors undergo apoptosis that is concomitant with the reduction and eventual loss of hematopoietic markers by the 20-somite stage (Ransom et al., 2004). The differentiation of lymphoid and myeloid cells is not affected in *mon* mutants. Most of *mon* homozygous animals die between 10 and 14 dpf, although rare mutant animals survive to adulthood. Analyses of hematopoiesis in the kidneys of these rare survivors revealed a severe block in erythroid differentiation at the proerythroblast stage (Ransom et al., 2004). Therefore, the *mon* gene is required during both primitive and definitive hematopoiesis specifically for erythroid differentiation. The defect gene in *mon* mutants encodes the zebrafish ortholog of the mammalian transcriptional intermediary factor 1γ (TIF1γ), a member of the TIF1 family of transcription cofactors (Ransom et al., 2004). The mechanism of TIF1γ function is largely unknown, but recent studies in Xenopus and in human cell culture have suggested an interaction between TIF1γ and TGF-β/BMP pathways (Dupont et al., 2005). Consistent with the blood defects in *mon* mutants, it has been found that the RNAi-mediated knockdown of TIF1γ in human hematopoietic progenitor cells inhibits erythroid differentiation in response to the TGF-β signal (He et al., 2006). Whether TIF1γ functions in a novel pathway or cooperates with classic erythropoietic factors such as *Gata-1* to regulate erythropoiesis is currently under investigation.

III. CLINICAL APPLICATIONS OF HEMATOPOIETIC STEM CELLS

Understanding the basic biology of blood development and HSC formation has paved the way for the clinical usage of HSCs. The capacity of HSCs to self-renew and ultimately give rise to all blood lineages makes them uniquely situated as a powerful tool for the treatment of a variety of blood diseases that are untreatable by traditional approaches. The best example is bone marrow transplantation, which has been used for the treatment of cancer-related hematopoietic deficiency and bone marrow failure states (Antin and Smith, 1995).

The transplantation of HSCs from adult bone marrow requires, first of all, the purification of HSCs. This is achieved by sorting HSCs on the basis of their unique surface marker profiles. Using monoclonal antibodies to select bone marrow cells on the basis of surface marker expression, both mouse and human HSCs can be properly isolated. All HSC activity in adult mouse bone marrow has been found in a population marked by the composite phenotype of *c-Kit*$^{+}$, *Thy-1.1*^{lo}, lineage markers$^{-/lo}$, and *Sca-1*$^{+}$ (Spangrude et al., 1988). These cells, when transplanted at the single-cell level, give rise to the long-term reconstitution of hematopoiesis in a lethally irradiated host. In humans, the combination of CD34^{+}, *c-Kit*$^{+}$, *Thy-1*^{lo}, and lineage markers$^{-/lo}$ cells resulted in the purification of the HSC population, with 85% to 95% purity

(Baum et al., 1992). In human bone marrow, however, HSCs are very rare. Only 0.5% to 5% of bone marrow cells are $CD34^+$ (Civin et al., 1990), and, of these cells, only 10% to 20% express the *Thy-1^{lo}, lineage marker$^-$* phenotype (Baum et al., 1992). To recover enough HSCs for transplantation, growth factors such as the granulocyte colony-stimulating factor (GCSF) are injected to stimulate the proliferation of HSCs and to mobilize HSCs out of the bone marrow and into the peripheral blood (Murray et al., 1995). The HSC-enriched mononuclear cell fraction can then be collected from the blood and sorted on the basis of the surface markers.

Isolated HSCs also provide good targets for gene therapy, which involves the introduction and expression of recombinant genes in somatic cells. For example, genetic hematopoietic disorders that are caused by mutation at a single locus can be treated by introducing a functional copy of the gene into the isolated HSCs using retrovirus-mediated gene transfer (Sutton et al., 1998; Case et al., 1999; Miyoshi et al., 1999) followed by the transplantation of these "corrected" HSCs back to the patient. Such gene therapy approaches have been performed in clinical trials on patients with severe combined immunodeficiency disease, and immune restoration has been observed after the transplantation (Bordignon et al., 1995).

Studies in different vertebrate models during the past few decades have significantly contributed to our understanding of the nature of HSCs. We believe that future studies using these model organisms will continually help us to understand the mechanisms by which HSCs differentiate into mature, functional cells. This will ultimately improve the use of HSCs for clinical applications.

SUMMARY

In this chapter, we reviewed the development of the vertebrate blood system. Using different model organisms, many factors controlling hematopoiesis have been identified, and a highly conserved genetic program is beginning to emerge.

- Vertebrate hematopoiesis occurs by a multistep process that begins with the induction of ventral mesoderm (see Figure 34.1). The BMP signaling pathway and its antagonists play important roles in patterning the mesoderm toward a ventral and hematopoietic fate.
- Endothelial and hematopoietic lineages are believed to derive from a common precursor called the *hemangioblast*.
- Primitive hematopoiesis occurs at extraembryonic YS blood islands in mammals or their equivalent in other species.
- Definitive hematopoiesis, which mainly occurs intraembryonically in the AGM region, produces definitive hematopoietic stem cells (HSCs) that ultimately give rise to all of the blood lineages throughout the life span.
- HSCs subsequently migrate through the circulation to colonize the newly forming hematopoietic organs (e.g., the FL [mouse, human, and Xenopus], the bone marrow [bird, mouse, and human], the kidney [zebrafish]) for further proliferation and differentiation into mature blood cells.
- Hematopoiesis is highly regulated by complex interactions among growth factors, cytokines, and transcription factors. Gene knockout studies in mice and the genetic mutants generated in zebrafish are powerful tools for identifying the essential genes that control hematopoiesis.

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GLOSSARY

Definitive hematopoiesis
The second wave of hematopoiesis that occurs shortly after the primitive hematopoiesis and that generates all blood lineages. In mammals, definitive hematopoiesis initiates in the aorta–mesonephros–gonads region.

Hemangioblast
An hypothesized common precursor for both blood cells and blood vessel endothelium cells.

Hematopoiesis
The developmental process by which various types of blood cells are formed.

Hematopoietic stem cells
The precursor cells that give rise to all types of blood cells. As stem cells, they are defined by their ability to self-renew and to form multiple cells types. In human adults, these cells are located in the bone marrow.

Primitive hematopoiesis
A transient wave of hematopoiesis that generates the first blood cells (mainly the red cells) in embryos. In mammals, the site of primitive hematopoiesis is the yolk sac.

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35 TOPICS IN VERTEBRATE KIDNEY FORMATION: A COMPARATIVE PERSPECTIVE

THOMAS M. SCHULTHEISS

Beth Israel Deaconess Medical Center and Harvard Medical School,
Molecular and Vascular Medicine Unit, Beth Israel Deaconess Medical Center, Boston, MA

INTRODUCTION

The kidney is a vital organ, and its main functions include the excretion of metabolic waste products and the maintenance of water balance. The basic functional unit of the vertebrate kidney is the *nephron* (Figure 35.1), which consists of three main components: a *glomerulus,* which filters the blood; a *tubule,* which reabsorbs substances from the glomerular filtrate and into which substances are secreted for excretion; and a *nephric duct,* which transmits the contents of the tubule to the exterior.

Although all vertebrate kidneys are comprised of nephrons, there is great variety in the morphology of the nephron and the arrangement of nephrons into kidneys, both among different species of vertebrates and in different kidney tissues within the same animal. All of these different types of vertebrate kidneys are produced by modifications of the kidney developmental program. Thus, studying kidney development in different vertebrates is important not only for what it can tell us about the kidney itself but also because it provides an excellent window into the question of how the basic building blocks of a tissue can be modified to create a great variety of forms.

Kidney development has been the subject of experimental embryologic study for more than 100 years, and, during the past 20 or so years, a large number of genes have been identified that play roles in kidney formation. It is not possible in this chapter to review comprehensively all of these studies; excellent classic and recent reviews and indeed whole books have been written about the topic, and the reader is referred to these for topics that are not included or covered in insufficient depth in this chapter (Dressler, 2002; 2006; Fraser, 1950; Goodrich, 1895; 1930; Lechner and Dressler, 1997;

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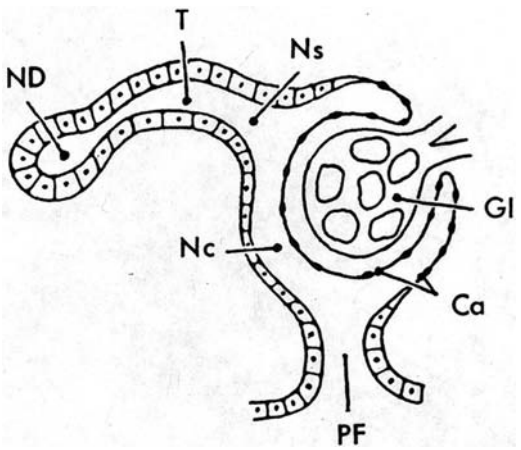

FIGURE 35.1 Schematic of a single nephron, illustrating its major components. Blood is filtered from capillaries at the glomerulus *(Gl)* across a specialized basement membrane in the kidney capsule *(Ca)*. Upon crossing the capsular membrane, the filtrate is now in the nephrocoele *(Nc)*, which is a subdivision of the body cavity or coelom. In the primitive situation, the nephrocoele is connected to the general coelom via a peritoneal funnel *(PF)*, but this connection is lost in many types of nephrons. From the nephrocoele, the filtrate passes through a nephrostome *(Ns)*, which is typically ciliated, and into the lumen of the kidney tubule *(T)*. In the tubule, substances are absorbed from the lumen and secreted into it by the epithelial tubular lining cells. Finally, the tubular contents drain into the nephric duct *(ND)*, from which they exit to the exterior. Typically the tubule is much longer and more convoluted than illustrated here. (Illustration from Torrey TW: Carnegie Institution of Washington Publication 603, Contributions to Embryology 35:175–197, 1954. Reprinted with permission of the publisher.)

Romer, 1955; Saxen, 1987; Vize et al., 1997; 2003; Yu et al., 2004). Rather than trying to restate that which has already been ably described, the current review will discuss vertebrate kidney development with a somewhat different emphasis from that of other recent reviews, namely from an evolutionary–developmental perspective. In other words, we will review the field with an eye toward trying to understand the basic developmental mechanisms that are common to the formation of all types of kidneys, as well as the mechanisms that have evolved to modify the structure of nephrons and their organization into kidneys. It is the author's belief that our understanding of the regulation of kidney development will be aided if we ask the following about each experimental finding: is this telling us something about kidney formation in general or about a specific type of modification in the kidney developmental program that has the effect of producing a specific type of kidney morphology? It will become clear in this chapter that we are only at the very beginning of being able fit what we currently know about the molecular regulation of vertebrate kidney development into an evolutionary–developmental context. It is hoped that by raising questions and pointing out areas in which our knowledge is lacking that this chapter may spur research and thought that broadens our understanding of kidney formation.

I. VERTEBRATE KIDNEY ANATOMY

We will first briefly review some of the most important morphologies of vertebrate nephrons and kidneys. The purpose of this section is to lay out

the variation displayed by vertebrate nephrons and kidneys that our models of kidney development will have to explain. The material is presented at the histologic level and largely without molecular data, because the main concepts and problems were already elucidated using purely morphologic criteria beginning approximately 100 years ago. During the later parts of this chapter, we will revisit many of these forms and concepts and attempt to connect them to more recent molecular and experimental data.

A. The Holonephros

Current consensus holds that the agnathostome fishes (jawless fishes), including the hagfish and lamprey, constitute a monophyletic outgroup to the remainder of the extant vertebrates (Takezaki et al., 2003), and thus features that are shared by agnathostomes and other vertebrates are good candidates for being conserved from the common ancestor that gave rise to all extant vertebrates. The kidneys of the embryonic hagfish and the apodans (legless amphibians) have a common, simple structure, and they are thus candidates for resembling the ancestral vertebrate kidney. The kidney of the hagfish embryo and the apodans extends throughout much of the length of the organism, and it contains one kidney tubule per segment; these are connected by a nephric duct that drains to the outside at the cloaca (Dean, 1899; Fraser, 1950; Goodrich, 1930; Price, 1897; 1904–1905; Romer, 1955). Because of its relative uniformity along the anterior–posterior axis, this type of kidney has been called a *holonephros*. The structure of the holonephros suggests that the vertebrate kidney is primitively a segmental organ.

Holonephric tubules consist of dorsal outpouchings from the embryonic coelom in a region between the somite and the lateral plate (the nephrocoele; Figures 35.2 and 35.3). At their dorsalmost aspect, each tubule bends posteriorly to join the next most posterior tubule, thereby connecting successive tubules with each other. These connecting regions comprise the nephric duct, which drains the urine produced by the tubules to the outside. In holonephric nephrons, the glomus, which is the vascular component of the kidney, is a segmental branch from the aorta that is associated with the ventral side of the nephric coelom (see Figure 35.2). As in many other anamniotes, the gloma are typically not intimately associated with the kidney tubules, because they are separated from them by the space of the nephrocoele. A connection called the *peritoneal funnel* also exists between the nephric and the lateral plate coela, thereby potentially allowing fluid in the lateral plate coelom to be taken up by the nephric tubules. Thus, if we consider the holonephros as being representative of the primitive vertebrate kidney, such a kidney would be expected to contain one kidney tubule per segment, with the tubules originating as outpouchings from the dorsal coelomic wall, with a nephric duct connecting the dorsal aspects of the tubules, and with a segmental vascular component associated with the ventral coelomic wall.

It should be noted that our knowledge of hagfish embryonic kidney anatomy is based on just a few series of histologic sections reported during the late nineteenth and early twentieth centuries, because hagfish embryos, which develop in the deep ocean, are extraordinarily hard to come by (Ota and Kuratani, 2006). There is no reason to think that the anatomic descriptions are inaccurate. However, because of the important position of the

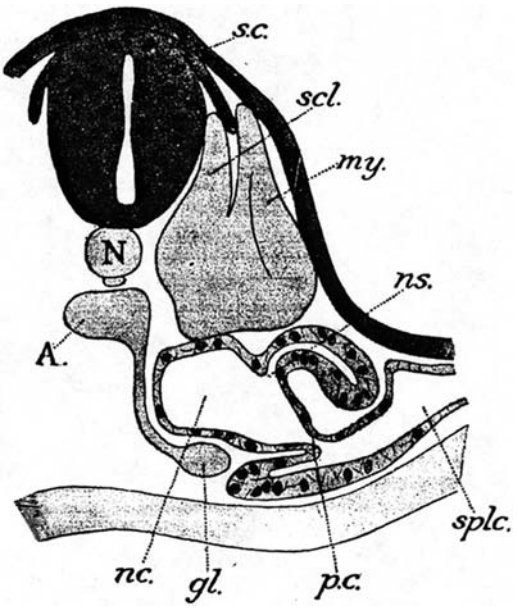

FIGURE 35.2 **Section through an apodan *(Hypogeophis)* embryo, illustrating features of the primitive nephron.** Note that the glomerulus *(gl)*, a branch from the aorta *(A)*, is separated from the entrance to the kidney tubule (the nephrostome, *ns*) by the relatively large space of the nephrocoele *(nc)*. *my,* Myotome; *N,* notochord; *pc*, peritoneal canal; *sc,* spinal cord; *scl,* sclerotome; *splc,* splanchnocoele. (Reproduced from Goodrich ES: Studies on the structure and development of vertebrates, London, 1930, Macmillan.)

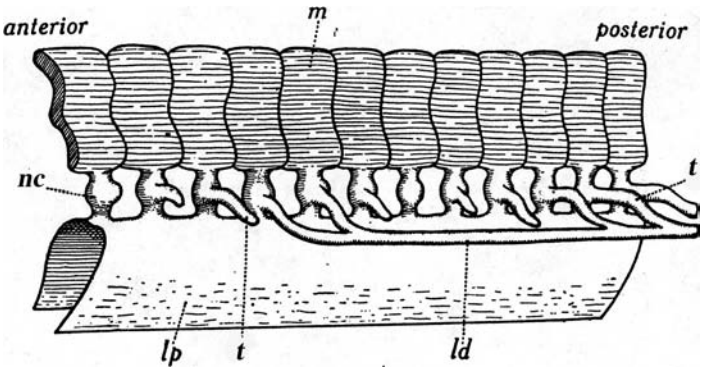

FIGURE 35.3 **Schematic illustrating the process of tubule and duct formation in an animal with one tubule per embryonic segment.** Tubules *(t)* bud out dorsally from the nephrocoele *(nc)* and bend posteriorly, joining up with tubules from more posterior segments to form the nephric duct *(ld)*. In the most primitive case (the holonephros), each segment of duct is formed by the posterior extension of the immediately anterior tubule. The current figure illustrates a more derived situation in which the duct is formed from a combination of contributions from posteriorly extending tubules as well as from the posterior extension of the duct itself. *lp,* Lateral plate; *m,* myotome. (Modified from Goodrich ES: Studies on the structure and development of vertebrates, London, 1930, Macmillan.)

hagfish in vertebrate phylogeny, it would be of great interest to have access to hagfish embryos and to be able to examine them using modern molecular and microscopic methods.

B. The Pronephros

The pronephros derives its name from its position in the anterior part of the organism and because of the fact that it develops first during embryonic development (Figure 35.4; Romer, 1955; Vize et al., 1997). It serves as the embryonic kidney of many fish and amphibians. As compared with the holonephros, the pronephros can be thought of as a differentiation of only the anteriormost part of the kidney developmental field. Although the anamniote pronephros is often small (consisting of only one pair of tubules in zebrafish and three pairs in Xenopus), it is functional, and zebrafish mutants that lack a pronephros die as embryos (Drummond et al., 1998). Birds and mammals also form a pronephros, but it has limited or no functionality, and it typically begins degenerating shortly after its formation (Fraser, 1950; Romer, 1955). As will be discussed later, the avian and mammalian pronephros appears to be evolutionarily conserved because pronephros formation is fundamentally linked to the formation of the nephric duct, which is essential for the development of the mesonephros and the metanephros. Thus, the avian/mammalian pronephros is a largely atavistic structure that is preserved because it serves an essential embryologic function.

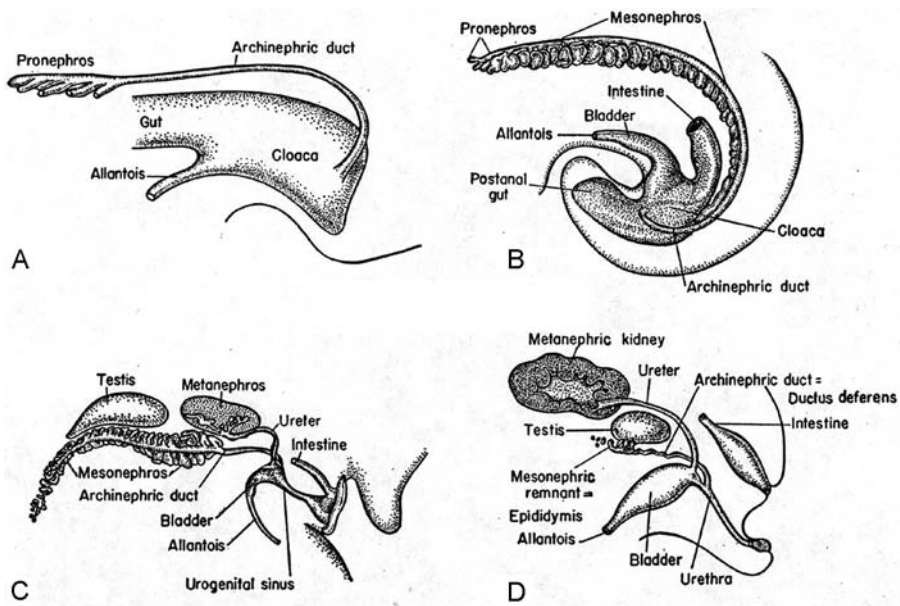

FIGURE 35.4 **Illustration of types of kidney tissue at different stages of a developing mammalian embryo. A,** Pronephros, with a few tubules draining into a nephric duct (here called the *archinephric duct*). **B,** The degeneration of much of the pronephros and the formation of a large mesonephros that extends over much of the trunk. **C,** The formation of the metanephros posterior to the mesonephros. **D,** The further development of the metanephros and the degeneration of the mesonephros, except for where it becomes associated with the testis. Note the single attachment of the metanephros to the nephric duct, which is in contrast with the multiple connections of the mesonephros. (Illustration from Romer AS: *The vertebrate body,* Philadelphia, 1955, WB Saunders.)

Because pronephric tubules form only in the anterior end of the embryo, the nephric duct in animals with a pronephros is typically quite long, because it extends from the pronephros to the cloaca. Recent investigations have found that some molecular markers that are found in the nephric duct of other vertebrate kidneys are found only in the distalmost part of the zebrafish nephric duct (Van Campenhout et al., 2006). Thus, the functional "tubule" of at least some embryos with a functional pronephros may extend to more posterior regions of the embryo than had been previously thought, and the true "nephric duct" of such embryos may only comprise the more posterior regions of the straight portion of the urinary system. The relation of the glomus to the tubule is variable in the pronephros. For example, in Xenopus, the glomus is separated from the entrance to the tubules by the space of the nephrocoele (Vize et al., 1997), as in the holonephric nephron (see Figure 35.2), whereas zebrafish pronephroi contain true glomeruli in which the vascular and tubule elements of the kidney are intimately associated (Drummond and Majumdar, 2003).

C. The Mesonephros and Metanephros

The mesonephros is the adult kidney of fish and amphibians and the functional embryonic kidney of birds and mammals. It lies posterior to the pronephros, and it usually extends over a larger portion of the trunk (see Figure 35.4). It typically contains multiple nephrons per body segment, with multiple connections of the these nephrons to the nephric duct (Cebrian et al., 2004). Mesonephroi typically contain true glomeruli, with an intimate association between the vascular and tubular components (Sainio, 2003).

Metanephroi are formed only in amniotes, where they serve as the adult kidney. The metanephros originates at the posterior end of the kidney morphogenetic field, although it can grow into more anterior locations, such as is seen in birds. In contrast with the mesonephros, the metanephros has only one or a few connections to the nephric duct (see Figure 35.4), which suggests that it may be derived from only one or a few embryonic segments. Despite its apparent origin from a small primordium, the metanephros can grow quite large, as would be expected for an organ that processes the metabolic and water balance needs of large adult animals. To achieve a large size despite having limited connections to the duct, the nephrons of the metanephros are highly branched, and they contain typical glomeruli (Fraser, 1950; Romer, 1955).

Having briefly reviewed the anatomy of the main vertebrate kidney forms, we will now turn to a discussion of the formation of these vertebrate kidney structures. We will focus on three areas that have received a significant amount of experimental attention: (1) the specification and early development of the kidney primordium; (2) the development of the nephric duct; and (3) the formation of the mammalian metanephros. Other topics will be discussed in a comparative manner as part of the treatment of the three main topics.

II. EARLY KIDNEY DEVELOPMENT AND SPECIFICATION OF THE INTERMEDIATE MESODERM

In the basic vertebrate body plan, the structure of the holonephros suggests that the region of mesoderm lateral to the somites is competent to form

nephric tissue and that this nephrogenic activity is uniform throughout a large segment of the anterior–posterior body axis. We will consider here two aspects of the establishment of this nephrogenic competence. First, we will discuss how the nephrogenic field is established along the medial–lateral (ML) (also referred to as the dorsal–ventral [DV]) axis of the mesoderm (i.e., why does kidney-forming tissue develop immediately lateral [or ventral] to the somites?). Because the location of the kidney-forming tissue along the ML axis is conserved throughout the vertebrates (an exception is in the zebrafish pronephros, which is certainly a specialized adaptation; this is discussed later), many of the factors that regulate the ML aspects of early kidney patterning are likely to be conserved among vertebrates. Second, we will consider how the nephrogenic field is established along the anterior–posterior (AP) axis. As discussed previously, kidney morphology along the AP axis is highly variable among different vertebrates and within individual animals, and alterations in the AP dimension of kidney formation are likely to play a role in the production of different types of kidneys.

A. The Medial–Lateral Axis of Early Kidney formation

The source of all kidney tissue is the intermediate mesoderm (IM), a region of mesoderm that lies between the somites and the lateral plate in the developing embryo (Figure 35.5). In the chicken embryo (as in mice and zebrafish), the earliest molecular marker of the IM is the putative transcription factor Odd1, the expression of which is initiated before the formation of a morphologically distinct IM (Figure 35.6; James et al., 2006; So and Danielian, 1999). Strictly speaking, Odd1 is not a specific IM marker, because its expression extends into the medial part of the lateral plate. In chicken and mouse embryos, Odd1 is expressed only in undifferentiated kidney precursor tissues; upon the differentiation of kidney tubules or duct, Odd1 is downregulated (James

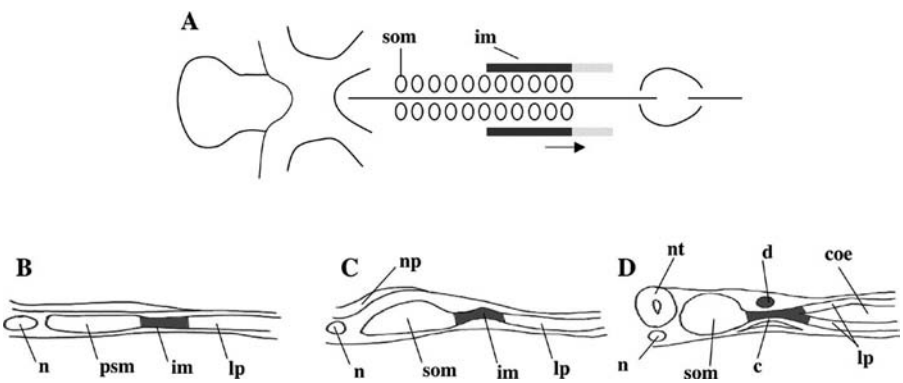

FIGURE 35.5 **Diagram of the intermediate mesoderm (IM) in the chicken embryo. A,** The IM is a strip of mesoderm that is located lateral to the somites. **B, C,** and **D,** The nephric duct rudiment forms when a portion of the IM bulges dorsally and separates from the IM. Subsequently, the nephric duct rudiment extends posteriorly and becomes epithelialized to form the nephric duct. Tubules subsequently form from the nephrogenic cord *(c)*. *coe,* Coelom; *d,* nephric duct; *im,* intermediate mesoderm; *lp,* lateral plate; *n,* notochord; *np,* neural plate; *nt,* neural tube; *psm,* presomitic mesoderm; *som,* somite. (Adapted from James RG, Schultheiss TM: Patterning of the avian intermediate mesoderm by lateral plate and axial tissues, *Dev Biol* 253:109–124, 2003.)

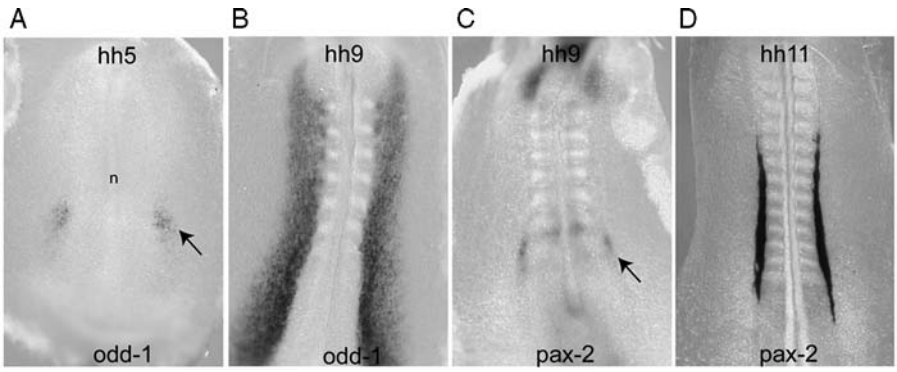

FIGURE 35.6 **Early gene expression in the chicken embryo intermediate mesoderm (IM). A,** Odd1 is expressed shortly after gastrulation in the nascent IM *(arrow)*. **B,** By Hamburger and Hamilton stage 9, Odd1 is expressed robustly in the IM and medial lateral plate. Its expression extends anteriorly until the axial level of the first somite. **C** and **D,** A second wave of IM gene expression begins at approximately Hamburger and Hamilton stage 9. At this stage, Pax2 is just beginning to be expressed. Pax2 expression is limited to the most medial aspect of the Odd1 expression domain, and it extends anteriorly only as far as the sixth somite axial level.
(Adapted from James RG, Kamei CN, Wang Q, et al: Odd-skipped related 1 is required for development of the metanephric kidney and regulates formation and differentiation of kidney precursor cells, *Development* 133:2995–3004, 2006; see color insert.)

et al., 2006). This is in contrast with other markers described later, which are maintained in differentiated kidney tissues. A second phase in IM gene expression begins in chicken embryos that are at Hamburger and Hamilton (HH) stage 8 (Hamburger and Hamilton, 1951), which occurs approximately 6 hours after the initiation of Odd1 expression, when the expression of the transcription factors Pax2 and Lim1 is detected in the most medial region (i.e., adjacent to the somites) of the Odd1 expression domain (see Figure 35.6; James and Schultheiss, 2003; Mauch et al., 2000). Unlike Odd1, Pax2 expression is confined to the IM and future kidney tissues, including both duct and tubular tissue, whereas Lim1 is initially expressed solely in the forming nephric duct (Schultheiss et al., 2003). At HH stage 10, Wt1 expression is first detectable in the IM and the most medial part of the lateral plate (James and Schultheiss, 2003). Zebrafish, Xenopus, and mouse also express Pax2 (or its paralog Pax8), Lim1, and Wt1 during early pronephros formation, which indicates that these genes are part of a shared vertebrate early kidney developmental program (Carroll et al., 1999; Dressler et al., 1990; Fujii et al., 1994; Kreidberg et al., 1993; Majumdar et al., 2000; Serluca and Fishman, 2001). In all of these species, Pax2 and Pax8 are expressed in the kidney tubules and duct, whereas Wt1 expression comes to be associated primarily with the forming glomerulus. In summary, IM and early kidney gene expression can be divided crudely into two stages. Phase 1 consists of Odd1 expression in the IM and the medial part of the lateral plate, whereas phase 2 consists of the activation of kidney-specific genes in the more medial sector of the Odd1-expressing domain.

The avian embryo has been used by a number of groups to gain insight into the timing of developmental commitment to a kidney fate along the ML axis and to determine the tissues and molecules that regulate such commitment. Transplantation experiments using chick–quail chimeras have determined that, when prospective pronephros cells reside in the primitive streak

(HH stages 4, 5, and 6), they are not committed to a kidney fate, because they will change fates if they are transplanted into prospective somite or lateral plate regions (James and Schultheiss, 2003; Psychoyos and Stern, 1996). Similarly, prospective somite and lateral plate regions of the streak will adopt a kidney fate if they are transplanted into the IM region of the primitive streak. During the approximately 6 hours after exit from the primitive streak, prospective pronephros cells become progressively resistant to respecification upon transplantation into non-IM regions of the embryo. Thus, by HH stages 8 and 9 (concomitant with the initiation of Pax2 and Lim1 expression in the IM), the prospective IM maintains Pax2 and Lim1 if it is transplanted into either the somite or lateral plate regions (James and Schultheiss, 2003).

The tissues and molecular factors that regulate the ML aspects of IM formation are just beginning to be uncovered. In the avian embryo, it seems clear at this point that signals from tissues that are both lateral and medial to the IM are important for specifying the IM. The first evidence that lateral signals could induce IM gene expression came from experiments in which lateral plate and somite tissue were combined in tissue culture. It was found that Pax2 was induced in the somite tissue, thereby indicating that a factor in the lateral plate could induce early kidney gene expression in paraxial mesoderm (James and Schultheiss, 2003). Subsequent experiments have found that bone morphogenetic protein (BMP) signaling is an important component of the lateral plate activity (James and Schultheiss, 2005). Purified BMP-2 or BMP-4 or constitutively active BMP receptors activate IM gene expression in the paraxial mesoderm both *in vivo* and *in vitro*, and the repression of BMP signaling using the BMP antagonist noggin represses IM gene expression in the IM. Interestingly, IM genes are activated only by specific levels of BMP signaling. At high levels of BMP signaling, lateral plate but not IM genes are activated, whereas lower levels of BMP signaling activate IM genes. This dose-sensitive effect of BMP signaling is cell autonomous (James and Schultheiss, 2005). Thus, one factor that regulates IM patterning is BMP signaling, with specific levels of BMP signaling being required to induce the expression of IM genes. One source of BMP signaling in the embryo is likely to be the lateral plate mesoderm itself, which expresses BMP-4, whereas levels of BMP signaling may be modulated by BMP antagonists expressed in midline and paraxial tissues (James and Schultheiss, 2003; Schultheiss et al., 1997). BMP signaling has also been implicated in the regulation of early kidney gene expression in zebrafish (Melby et al., 2000). In Xenopus, the ectopic expression of a combination of Lim1 and Pax8 leads to the production of ectopic pronephric tubules (Carroll and Vize, 1999). Interestingly, these ectopic tubules appear to be generated only in the region of the prospective somites, thereby suggesting that the paraxial mesoderm is more permissive for kidney formation than the more ventral regions of the embryo. This is consistent with the data from chickens, and it suggests that conditions in the lateral plate (with its high levels of BMP signaling) may be nonpermissive for kidney development (Figure 35.7; James and Schultheiss, 2005).

However, BMP signaling is almost certainly not the only important signal that regulates IM gene expression. Several laboratories have found evidence for a midline or paraxial signal that promotes IM gene expression in both avian and amphibian embryos. The blockage of communication between the dorsal and intermediate regions of the mesoderm prevents the activation of IM genes in chicken embryos (Barak et al., 2005; Mauch et al., 2000), and

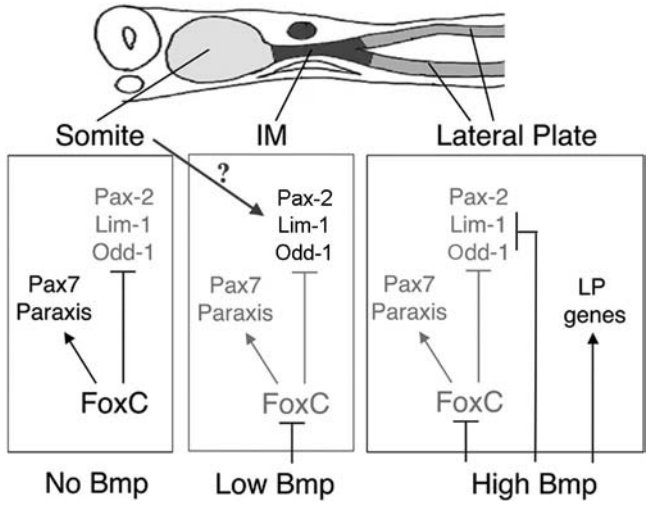

FIGURE 35.7 **Model of factors regulating intermediate mesoderm (IM) formation.** In the somite, IM genes are repressed by somite transcription factors such as FoxC1 and FoxC2. Bone morphogenetic protein signaling in the IM region represses somite transcription factor gene expression and derepresses the expression of IM genes such as Odd1. An unknown factor or factors that likely originate from axial or paraxial tissues is required for the robust expression of other IM genes such as Pax2 and Lim1. In the lateral plate, high levels of bone morphogenetic protein signaling repress IM gene expression through unknown molecular mechanisms. (Modified from James RG, Schultheiss TM: Bmp signaling promotes intermediate mesoderm gene expression in a dose-dependent, cell-autonomous and translation-dependent manner, *Dev Biol* 288:113–125, 2005).

dorsal structures have also been found to promote kidney tubule formation in Xenopus (Seufert et al., 1999). Such a dorsal signal is unlikely to be simply a BMP antagonist, because transplants of Hensen's node (the most dorsal embryonic structure) into the lateral plate can induce IM gene expression at a stage of embryonic development at which implants of noggin-expressing cells do not have this effect (James and Schultheiss, 2003, 2005). In addition, *in vivo* and *in vitro* studies have found that BMP signaling is more efficient for the induction of the expression of Odd1 than it is of more specific kidney markers such as Pax2 and Lim1 (James and Schultheiss, 2005). Because Pax2 and Lim1 expression is activated after Odd1 and only in the region immediately adjacent to the somites, it is likely that an additional signal emanating from midline or paraxial tissues is required for the expression of the full set of IM genes. The molecular nature of this dorsal signal (or signals) is not currently known, but it is under active investigation. *In vitro* experiments with Xenopus animal caps have found that a combination of activin and retinoic acid (RA) signaling promotes the generation of kidney tubules (Moriya et al., 1993). Although both activin-like (Schier, 2003; Sive, 1993) and RA (Swindell et al., 1999) signals are associated with dorsal embryonic structures, it is not clear how the *in vitro* findings relate to the requirement for dorsal signaling or to the generation of kidney tubules *in vivo*. One of the effects of activin in the *in vitro* animal cap experiments is likely to be mesoderm induction, but that does not rule out more kidney-specific roles for activin-like signaling during later stages of development. It is of interest that activin and RA have

also been found to promote kidney gene expression in mouse embryonic stem cells (Kim and Dressler, 2005).

One important area of current research is attempting to make connections between the signals that induce IM formation and the activation of IM-specific genes. Misexpression in the IM of the forkhead family members FoxC1 and FoxC2, which are normally expressed in the somites, leads to the repression of IM gene expression on a cell-autonomous basis, whereas complementary experiments have found that, in mouse FoxC1 and FoxC2 knockouts, IM gene expression is expanded into the somite region (Wilm et al., 2004). Taken together, the FoxC1, FoxC2, and BMP data have been combined into a working model in which IM gene expression is under negative regulation by somite transcription factors and in which BMP signaling (at the appropriate dose) represses somite gene expression in the IM and thereby derepresses IM gene expression (see Figure 35.7; James and Schultheiss, 2005). One major aim for future research will be to identify other signals, including those from dorsal sources, that induce a subportion of the Odd1-expressing region to express kidney-specific markers, such as Pax2, Pax8, Lim1, and Wt1.

B. The Anterior–Posterior Axis of Early Kidney formation

Many aspects of kidney morphology vary along the AP axis, including the following:

(a) There is an anterior limit to the portion of the IM that goes on to form kidney tissue. In the chicken embryo, this limit lies at the axial level of somite 6 (Barak et al., 2005), whereas, in Xenopus, it lies at approximately somite 3 (Vize et al., 1997).
(b) The pronephros, mesonephros, and metanephros are located at different locations along the AP axis, and they have very different morphologies.
(c) In many vertebrates, only anterior IM is capable of giving rise to nephric duct tissue (this topic is discussed further in the next section).

It is likely that interactions of the basic kidney developmental program with factors that pattern the AP axis underlie the different kidney developmental fates along the AP axis.

Much less is known about the AP dimension of kidney formation than is known about the ML dimension. Hox genes are good candidates for regulating at least some of these aspects of kidney AP patterning. Members of the Hox11 paralogous family are expressed in the metanephros but not in more anterior kidney tissue, and the loss of Hox11 function results in the absence of the metanephros (Patterson et al., 2001; Wellik et al., 2002). The anterior border of the pronephros and the posterior border of the duct-forming region correlate with the expression boundaries of particular Hox genes (H. Barak, R. James, R. Reshef, and T. M. Schultheiss, unpublished data), but the functional significance of this correlation has not yet been established.

Experiments in the chick embryo have investigated the time at which the AP pattern in the IM is established and the factors that regulate such patterning (Barak et al., 2005). Normally, IM adjacent to somites 1 though 5 in the chick embryo expresses Odd1, but it will never express specific kidney markers such as Pax2 or Lim1. While it is still residing in the primitive streak,-prospective IM that is fated to lie adjacent to somites 1 through 5 can be

induced to express kidney markers if it is transplanted into an older primitive streak that gives rise to the IM adjacent to somites 6 and those posterior to it. As with ML patterning, prospective anterior and posterior IM tissues become fixed to their respective identities during the course of their migration into the IM region. Interestingly, if prospective anterior IM residing in the primitive streak is transplanted directly into mature IM adjacent to somites 1 through 5 (thereby eliminating its normal migration path), the transplanted tissue goes on to express kidney markers ectopically. These data suggest that kidney-inducing signals are present throughout the AP axis of the embryos (and not just at the axial levels at which the kidneys form). The process of the migration of cells from the primitive streak into the IM of axial levels 1 through 5 appears to expose cells to factors that inhibit their ability to respond to kidney-inducing signals, thus restricting kidney gene expression to the axial levels of somite 6 and of those posterior to it.

Before leaving the topic of early kidney patterning, it is worth noting that the relationship between the ML and AP axes of the early embryonic kidney is somewhat different in chicken and mouse embryos as compared with, for example, zebrafish embryos. In birds and mammals, the tubule, glomerulus, and duct primordia of each nephron are located at the same axial levels but at different regions of the ML axis. This is likely to be a conserved feature from the primitive vertebrate state, because it is shared with the holonephros (see Figure 35.2). In the zebrafish pronephros, on the other hand, the glomus primordia (as marked by Wt1 expression) is located more anteriorly than other regions of the pronephros (Serluca and Fishman, 2001). The Pax2 domain partly overlaps the Wt1 domain and extends more posteriorly, and it is later associated with the tubule and anterior duct, whereas a more posterior Sim1 domain is later associated with the duct. Thus, in zebrafish, cell fates within the nephron are deployed along the AP axis, and this is unlike the situation in the bird and mammal pronephros. These differences may have their origin in the modification in the fish pronephros of an ancestral kidney differentiation pattern as compared with its higher degree of conservation in birds and mammals. Regardless of how the difference evolved, it is important to be aware of these different patterns when comparing developmental mechanisms in the different species. In particular, factors that regulate glomus as compared with tubule formation might be expected to be associated with AP positioning in the zebrafish but with DV positioning in amniote embryos.

III. FORMATION OF THE NEPHRIC DUCT

The development of the nephric duct is an important topic for a number of reasons. It is one of three main structural units of all kidney types (the others being the tubules and the glomus/glomerulus), and studies of nephric duct formation can yield insights into how the different components of the kidney are differentiated with respect to each other. From an evolutionary perspective, the formation of the nephric duct is quite variable across vertebrate groups, and its study offers the opportunity for insights into the developmental mechanisms that account for such variability. In many species, the nephric duct undergoes a remarkable migratory process that is still poorly understood at the molecular level. Finally, as will be discussed in the section about the

metanephros, the nephric duct plays an essential role in the induction of the metanephric (as well as the mesonephric) tubules.

To gain some evolutionary context, we begin our discussion of the nephric duct by again considering the development of the holonephros. It must be stressed again that our knowledge of holonephros nephric duct formation is derived from serial histologic sections of a very limited number of specimens; thus, it is urgently in need of a revisit. That being said, classic descriptions of holonephros nephric duct formation hold that the dorsalmost part of the kidney tubule in each body segment bends posteriorly and merges with the tubule of the next most posterior segment (see Figure 35.3; Fraser, 1950; Price, 1897; 1904–1905). These connections between tubules collectively make up the nephric duct. Thus, each segment of the embryo that contains a kidney tubule also generates a segment of the nephric duct. The formation of the most posterior portion of the duct, from the most posterior tubule to the opening through which the duct drains to the outside, has not been described.

When we move to the pronephros, a problem arises: if duct segments form only where there are tubules (as in the holonephros), how can a duct be generated that drains urine from the pronephros at the anterior end of the embryo to the exit at the posterior end? It appears that at least two developmental mechanisms have evolved to solve this problem. The most highly studied mechanism is seen in amphibian embryos as well as in avian and mammalian embryos. In these species, a nephric duct rudiment forms in the vicinity of the pronephros and proceeds to extend posteriorly to where it drains into the cloaca or bladder (Schultheiss et al., 2003). In the chicken, the duct rudiment forms as a dorsal outcropping from the intermediate mesoderm; this is reminiscent of the dorsal extensions that give rise to the holonephros nephric duct (see Figure 35.5; James and Schultheiss, 2003). The fact of duct migration has been convincingly demonstrated in both amphibian and avian embryos by fate mapping and microsurgical techniques (Obara-Ishihara et al., 1999; Poole and Steinberg, 1981; Schultheiss et al., 2003).

There is some evidence that an alternative mechanism may contribute to the process of nephric duct formation in zebrafish and Xenopus embryos. In Xenopus, it has been reported that cells in the posterior trunk of the embryo can participate in duct formation (Cornish and Etkin, 1993), although in this setting there is also a contribution from the anterior migrating duct rudiment. This is unlike the situation in the chick, in which all duct tissue appears to originate in the anterior duct rudiment (Obara-Ishihara et al., 1999; Schultheiss et al., 2003). In one study in zebrafish, the labeling of cells in the flank of the embryo did not uncover any evidence for the migration of the duct (Serluca and Fishman, 2001). One caveat to this experiment is that it does not rule out duct migration at an earlier stage of development. In any case, these studies in Xenopus and zebrafish should be followed up, because they indicate that, in at least some species, it may be possible to generate duct tissue without any connection to tubular tissue.

Despite many decades of study, the molecular mechanisms that regulate duct rudiment formation and extension are still obscure (Schultheiss et al., 2003). Studies in Xenopus have implicated Notch signaling in the allocation of the pronephric rudiment into duct and tubule primordia (McLaughlin et al., 2000). In the chicken, BMP signaling is required for the epithelialization of the duct rudiment but not for the posterior migration of the duct primordia (Obara-Ishihara et al., 1999).

Despite the differences between holonephric and pronephric nephric duct formation, there may be some fundamental similarities. In both situations, there appears to be a connection between duct and tubule formation, such that the duct forms as a dorsal extension from a region that is forming (e.g., the holonephros) or that will form (e.g., the pronephros) kidney tubules. The difference between the two situations is that, in the pronephros, the connection between duct and tubule formation is limited to the pronephric region, whereas, in the holonephros, the two tissue types are linked throughout the length of the body axis. It is of interest that, in birds and mammals, in which the pronephros is essentially nonfunctional, the nephric duct forms in association with rudimentary pronephric tubules. Thus, there may be an essential link between pronephric tubule and nephric duct formation, with the nonfunctional pronephric tubules being a byproduct of the need to generate a nephric duct. It is not clear whether the migration of the nephric duct rudiment is an evolutionary innovation or rather if it is an elaboration of the basic tendency of the dorsalmost regions of the kidney tubules to extend posteriorly, as is seen in the holonephros.

In amniote embryos, the IM of the mesonephric and metanephric regions does not appear to have any ability to generate nephric duct tissue. Thus, blocking the migration of the duct rudiment in chicken embryos leads to the absence of a duct posterior to the blockade (Gruenwald, 1937; Waddington, 1938). One caveat to interpreting this finding is that the nephric duct is itself required for the induction of mesonephric and metanephric tubule formation (as is discussed later). Thus, if tubule formation is required for duct formation, then the essential precursors for duct formation would not be generated in embryos in which duct migration has been blocked.

The main contribution of studies in the mouse embryo to the understanding of nephric duct formation has come from genetics. Abnormalities in the formation of the nephric duct are seen in many mouse mutants, including Odd1, Pax2, Pax8, Lim1, and Gata3 (Bouchard et al., 2002; Grote et al., 2006; James et al., 2006; Shawlot and Behringer, 1995). However, because of the mutual interactions between duct and tubulogenic mesenchyme (discussed in the next section) and because many of these genes are expressed in both the duct and the prospective tubules, it is usually unclear whether these genes are required autonomously in the duct. One exception is Gata3, which is not expressed outside of the duct during early kidney formation (Grote et al., 2006); thus, it appears to play a specific role in duct formation. Gata3 mutants do form a duct, but it exhibits abnormalities in posterior extension and morphogenesis. The absence of a significant number of genes that are required specifically for duct formation and that are not also required for other aspects of kidney formation should maybe not be surprising given the common origin in the IM of both the duct and the tubules and the intimate relationship between duct and tubule formation throughout kidney development.

IV. FORMATION OF THE MAMMALIAN METANEPHROS

We will now turn to the metanephros, which is the most-studied component from a molecular standpoint, not only of the mammalian nephric system, but of vertebrate nephrogenesis in general. Studies of metanephros development

are the main source of knowledge regarding the genetics of vertebrate kidney development. However, again we must bear in mind that the metanephros is a specialized structure, and thus not all of the developmental pathways that regulate metanephros formation will be found to be involved in the formation of other types of kidneys. During this discussion, we will introduce comparisons between metanephric and other forms of kidney development for which such data exist to attempt to determine which features of metanephric development appear to be conserved in other types of vertebrate kidney development and which appear to be specializations that are specific to metanephros formation.

A. Overview

The first morphologic evidence for metanephros formation is the appearance of a bud called the *ureteric bud* (UB) near the posterior end of the nephric duct. The UB invades the adjacent intermediate mesoderm, which at this point is called the *metanephric mesenchyme* (MM; Figure 35.8). As a result of reciprocal interactions between the UB and the MM (Dressler, 2006; Lechner and Dressler, 1997; Yu et al., 2004), the UB branches, and cells of the MM condense at the tips of the branching UB. The condensed MM patches give rise to tubules and glomeruli, whereas the branched UB gives rise to the collecting system of the kidney. After multiple rounds of branching and condensation, the result is a highly organized arrangement of thousands of nephrons (approximately 10,000 in the mouse and 1,000,000 in the human metanephros) that drain into a central ureter (Saxen, 1987).

From a purely morphologic point of view, the metanephros differs from the mesonephros in its highly branched structure and single ureteral drainage as compared with the mesonephros, which is much less highly branched and which has multiple connections to the nephric duct. Although the metanephros is typically larger than the mesonephros, it appears that the region of the IM (the MM) that gives rise to the metanephros extends the length of only a few somites, whereas the mesonephros derives from a much larger extent of trunk IM. Thus, the production of a large metanephros required the evolution

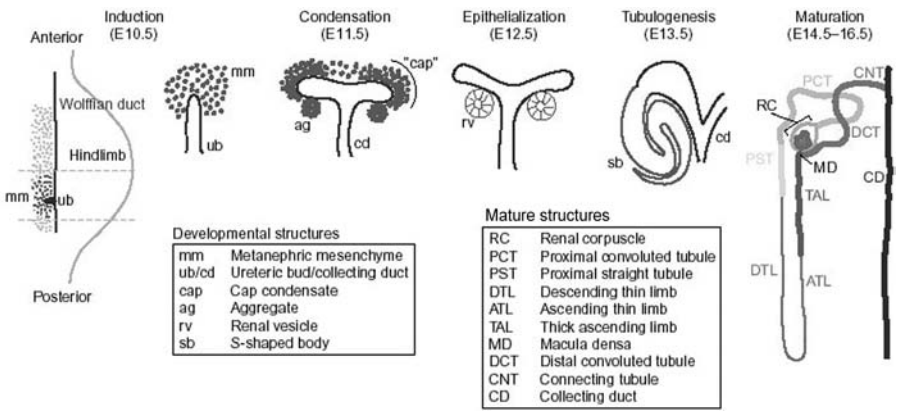

FIGURE 35.8 **Steps in the formation of mouse metanephric nephrons.** (Figure originally published in Yu J, McMahon AP, Valerius MT: Recent genetic studies of mouse kidney development, *Curr Opin Genet Dev* 14:550–557, 2004. Reprinted with permission of the publisher. See color insert.)

of a mechanism for greatly expanding the nephrogenic mesenchyme while generating nephrons. It is this tremendous expansion of the nephrogenic mesenchyme that underlies many of the features of kidney development that are specific to the metanephros.

B. The Ureteric Bud

The first morphologically recognizable step in metanephros formation is the formation of a bud from the nephric duct: the UB. Formation of the UB is dependent on the signaling of the Gdnf/cRet pathway, with Gdnf expressed in the MM and its receptor cRet expressed in the nephric duct (Moore et al., 1996; Sainio et al., 1997b; Sanchez et al., 1996; Schuchardt et al., 1995). Mutations in either Gdnf or cRet lead to the absence of UB formation. Because cRet is expressed throughout the length of the nephric duct and Gdnf is also expressed in more anterior regions of the embryo, the question arises as to why only one UB forms and why it forms in the region of the MM. The examination of the Gdnf expression pattern sheds some light on this issue. Just before the time of UB formation, Gdnf expression becomes restricted to a region that is adjacent to the future UB site. This restriction is regulated at least in part by a mechanism involving FoxC1 and FoxC2 as well as Robo2/Slit2. In mutants of FoxC1 and FoxC2 or Robo2/Slit2, Gdnf remains expressed in more anterior IM regions, and multiple UBs are formed (Grieshammer et al., 2004; Kume et al., 2000). Thus, it appears that a broad region of the nephric duct is competent to form UBs and that the restriction of the ligand Gdnf is important for the generation of a single UB. Consistent with this interpretation is the fact that ectopic Gdnf can cause multiple buds to form from a nephric duct in vitro (Sainio et al., 1997b). Although the restriction of Gdnf provides an explanation for how a single UB is generated at this particular time in development, this does not explain why ectopic UBs do not form at earlier times, before Gdnf expression has become restricted. One possibility is that other, currently unknown components of the UB induction pathway are not functional until after embryonic day 10.5, thus preventing the premature initiation of UB formation.

C. The Metanephric Mesenchyme Before its Interaction with the Ureteric Bud

The mouse MM forms as an expanded region of IM at the axial level of the hind limb. On embryonic day 10.5, before its interaction with the UB, the MM is not readily distinguished by morphologic criteria. However, already at this time, the MM exhibits important molecular characteristics, and it expresses a significant number of kidney-associated regulatory genes, including Odd1, Eya1, Six1, Six2, Sall1, Gdnf, Wt1, and Pax2 (Armstrong et al., 1993; Brodbeck and Englert, 2004; Dressler et al., 1990; Kalatzis et al., 1998; Moore et al., 1996; Nishinakamura et al., 2001; Ohto et al., 1998; Pichel et al., 1996; Sanchez et al., 1996; So and Danielian, 1999; Xu et al., 2003). This is an important point, because it indicates that the MM has been significantly patterned before its interaction with the UB and that it does not require interaction with the UB for the initial expression of kidney-specific genes. It is likely (although not yet demonstrated) that the mechanisms that regulate initial IM formation (including BMP signaling, as discussed previously) are also involved in the early specification of the MM. Most genes that are characteristic of the MM at this time, such as Eya1, Six1, Six2, Sall1, Gdnf,

Odd1, Wt1, and Pax2, are not confined to the MM; they are also being expressed in more anterior regions of the IM. As discussed previously, among the few exceptions are genes of the Hox11 paralogous cluster, which are expressed in the MM region but not in more anterior IM (Patterson et al., 2001; Wellik et al., 2002). Because compound mutants in the Hox11 group do not generate a metanephros, it is possible that Hox11 family members play a role in specifying the metanephric character of this region of the IM.

Many of the genes expressed in the MM before its interaction with the UB have been studied in mouse mutations. From these studies, Odd1 appears to lie genetically upstream of most genes that are known to be expressed in the MM, because Odd1 mutants exhibit an absence of Gdnf, Pax2, Eya1, Sall1, and Six2 (James et al., 2006; Wang et al., 2005). One exception is Wt1, which is expressed relatively normally in Odd1 mutants (James et al., 2006). Eya1 is required for the expression of other known MM genes aside from Odd1 and Wt1 (Sajithlal et al., 2005; Xu et al., 1999), and Six1 is required for Pax2, Six2, and Sall1 expression (Xu et al., 2003). Recent data from mouse metanephros cultures indicate that Wt1-regulated vascular endothelial growth factor signaling within the MM is also important for the activation of a subset of early metanephric genes (Gao et al., 2005). In mutants of Odd1, Wt1, and Eya1 (and of course of Gdnf), the UB does not form, whereas mutants for Six1 begin to form a UB (Xu et al., 2003), and Sall1 mutants show an arrest of UB formation at a somewhat later stage (Nishinakamura et al., 2001). Thus, the beginnings of a molecular pathway for early MM gene expression are emerging, with Odd1 lying at the most upstream point and being followed by Eya1, then Six1, and then Pax2, Six2, and Sall1. The expression of Wt1, which in turns regulates vascular endothelial growth factor expression, appears to be regulated largely independently of this pathway. It must be noted, however, that certain aspects of the regulatory relationships between these genes do not fit easily into simple hierarchical patterns; the interactions among gene products are likely to be complex.

Although most genetic studies of mouse kidney development have focused on the metanephros, some of these studies have also provided information about the genes required for mesonephros formation. Some genes that are expressed in both mesonephric and metanephric mesenchyme are apparently required for metanephric but not mesonephric kidney development. Thus, embryos lacking Eya1, Six1, and Gdnf have apparently normal mesonephroi (Moore et al., 1996; Nishinakamura et al., 2001; Sajithlal et al., 2005; Sanchez et al., 1996; Xu et al., 1999; 2003), whereas embryos lacking Odd1 and Wt1 have defective (although present) mesonephroi (James et al., 2006; Sainio et al., 1997a). One possible explanation for the apparent lack of requirement for some of these genes in mesonephros formation is the existence of redundant pathways in mesonephros but not metanephros development. Another possible explanation is that most genetic studies have focused on the examination of the metanephros. Thus, subtle alterations in the mesonephros may have been missed in some cases. In this context, it should be noted that the normal mesonephros in the mouse is very small, containing only a dozen or so tubules, and it is thus atypical as compared with other amniotes. Therefore, it is possible that genes that appear to be dispensable for mouse mesonephric development may actually be required for the normal development of a more substantial mesonephros. Most interesting is the

possibility that genes such as Eya1, Six1, and Gdnf, although expressed in the mesonephros and metanephros, are truly required only for metanephric development. This is consistent with the possibility (discussed later) that there are distinct regulatory pathways that operate in the metanephros and that function to generate the tremendous degree of controlled growth that is characteristic of the metanephros.

D. Differentiation of the Metanephric Mesenchyme

At the onset of interaction with the UB, gene expression in the MM is relatively uniform. After the initiation of UB formation and branching, gene expression in the MM becomes localized to specific subdomains (see Figure 35.8). MM condenses adjacent to the branching UB to form a "cap" of cells (Cho and Dressler, 2003; Dressler, 2002). Cells of the cap express Odd1, Eya1, Six2, Gdnf, Pax2, and other genes (Dressler et al., 1990; James et al., 2006; Kalatzis et al., 1998; Sainio et al., 1997b), although it is not clear whether all of the cap cells express all of the genes simultaneously.

The next phase of MM differentiation is the further condensation of the region of the cap closest to the UB tip to form pretubular aggregates, which typically form on the side of the UB closest to the ureteric stalk (see Figure 35.8). The pretubular aggregates then epithelialize to form epithelial vesicles (EVs), which are the progenitors of the nephric tubules and glomeruli. It is worth noting that the formation of the EVs is an example of mesenchymal-to-epithelial transition, which is much rarer than its opposite, epithelial-to-mesenchymal transition. There are several lines of evidence that indicate that Wnt signaling is important in the progression from the condensed mesenchyme of the caps to the formation of pretubular aggregates and EVs. The first evidence came from experiments in which MM was cultured in organ culture. During the 1950s, Grobstein et al. (1955) found that, if the MM is placed into culture, it will not undergo differentiation; however, if it is cocultured with the UB, it will differentiate into epithelialized tubules. In 1994, it was reported that fibroblasts expressing Wnt1 can substitute for the UB (Herzlinger et al., 1994). However, Wnt1 is not expressed in the UB, so the translation of this *in vitro* finding to kidney development *in vivo* was not clear. Subsequently, it was found that Wnt4 is expressed in pretubular aggregates and that the loss of Wnt4 prevents the subsequent development of the aggregates into kidney tubules (Kispert et al., 1998). Recently, it was reported that Wnt9b is expressed at the tips of the branching ureter and that, in embryos carrying Wnt9b mutations, MM differentiation is arrested before the pretubular aggregate stage (Carroll et al., 2005). Thus, a picture is emerging in which Wnt signaling is required during at least two phases in MM differentiation: a Wnt9b signal from the UB tips is required for the formation of pretubular aggregates, and a Wnt4 signal originating from the aggregates themselves is required for further tubular differentiation. Although Wnt9b and Wnt4 originate from different tissues, it could be argued that they both are part of a continuing requirement for Wnt signaling to allow MM differentiation to proceed. One possible model that is currently not proven is that the condensed cells of the cap may be blocked in their differentiation and that the function of Wnt signaling would be to derepress kidney differentiation. The function of carefully deployed Wnt signaling in this model would be to allow for the selective differentiation of parts of the MM into tubules

while other regions of the MM are maintained in a replicating, undifferentiated state to provide more material for subsequent rounds of tubule formation. Some tangential supporting evidence for this type of model comes from experiments in which the overexpression of Odd1 leads to the inhibition of epithelial kidney tubule formation (James et al., 2006). Support for the idea that differentiation of the condensed mesenchyme is under active suppression comes from the recently published Six2 mutant which undergoes precocious metanephric tubule formation, implying that Six2 is involved in the maintenance of condensed mesenchyme in a non-epithelialized state (Self et al., 2006).

After its formation, each EV undergoes a complex process of regionalization and growth to generate a kidney tubule and glomerulus. On a morphologic level, the EV expands to form first a "comma-shaped body" and then an "S-shaped body," which fuses (by a poorly understood mechanism) with a branch of the UB (Cho and Dressler, 2003). On a molecular level, the EV shows some signs of regionalization shortly after its formation, with respect to Notch signaling and integrin gene expression (Cheng et al., 2003; Cho and Dressler, 2003). Fibroblast growth factor signaling is critical for proper EV growth and patterning; conditional fibroblast growth factor 8 mutants show a loss of the loop of Henle and other nephron patterning defects (Grieshammer et al., 2005). Notch signaling also appears to be important in the patterning of the EV, because loss-of-function Notch mutations result in a loss of proximal tubule and glomerulus formation (Cheng et al., 2003). The picture that is beginning to emerge is that initial patterning of the tubule takes place already at the EV stage and that an important element of tubular differentiation is the selective growth of portions of this patterned rudiment. BMP-7 signaling has also been implicated in the subsequent growth of the metanephric kidney (Dudley et al., 1995).

Subsequent growth and differentiation of the metanephric mesenchyme include further differentiation of the glomerulus, specialization of the tubular tissue, the formation and differentiation of the stroma (which lies between the nephrons), and the differentiation of the vascular system of the kidney. Most of these processes are not yet well understood at the molecular level, but the interested reader can find summaries of current knowledge in recent reviews (Abrahamson and Wang, 2003; Cho and Dressler, 2003; Cullen-McEwen et al., 2005; Woolf and Yuan, 2003; Yosypiv and El-Dahr, 2005).

E. Further Differentiation of the Ureteric Bud and Collecting System

When we left the story of the UB, it had just branched from the nephric duct in response to Gdnf signals emanating from the MM. Subsequently, the UB undergoes a large series of branching divisions that result in the highly branched collecting system of the metanephros. Although these branching events have often been described as bifurcations, in fact many different types of branching patterns have been observed (Cebrian et al., 2004). Mosaic mice in which green fluorescent protein is expressed in scattered cells in the UB have been used to trace the movement of cells within the UB as it branches (Shakya et al., 2005). These studies have found that the green-fluorescent-protein–labeled cRet$^{-/-}$ cells can participate in UB formation and branching if they are accompanied by wild-type cells, but that such mutant cells cannot move into the ampullae of the collecting ducts, where future branching events will take place. Thus, cRet signaling may be required not for collecting duct

formation *per se* but rather for the ability to undergo budding and branching events. While signals from the MM are required for the induction of a UB and for subsequent branching of the UB *in vivo*, the UB can undergo extensive branching *in vitro* in the absence of the MM (Qiao et al., 1999). The culture medium in these experiments contained conditioned medium from MM cultures. These findings suggest that the UB itself possesses an intrinsic ability to undergo branching morphogenesis in response to general environmental cues from the MM. After UB formation, Gdnf expression becomes confined to the MM adjacent to the branching points of the UB (Miyamoto et al., 1997). This localized Gdnf expression has often been interpreted as inducing branching in the UB. However, the data indicating that the UB can branch in response to a nonlocalized Gdnf signal require some revisiting of this interpretation, and they suggest that the function of Gdnf and other localized signals in the MM might be to modify an autonomous branching program in the UB. The mechanisms of renal branching morphogenesis have been the subject of a large number of studies, which have been recently reviewed (Costantini, 2006).

F. Concluding Remarks Concerning Metanephros Development

As discussed above, the formation of the mammalian metanephros is an iterative process that leads to the generation of multiple generations of nephrons. This requires an intricate balance between the maintenance and proliferation of precursor tissue on the one hand and the differentiation of nephrons on the other. An important but poorly understood issue concerns how this balance between proliferation and differentiation is maintained, and the reason that the kidney stops growing and generating new nephrons after it reaches a certain size (which occurs approximately 3 weeks after birth in mice) also needs to be addressed. At this time, condensed mesenchymal cells can no longer be seen at the periphery of the kidney, which is the zone in which new nephrons are generated. The assumption has been that, at this time, all kidney precursor cells have been depleted; however, this has not been conclusively demonstrated. It would be interesting to determine whether any markers that are characteristic of MM cells are expressed in older kidneys and, if so, to attempt to characterize these cells.

SUMMARY AND SYNTHESIS

The preceding discussion has reviewed the formation of selected components of the nephric system in several different vertebrate species. In this concluding section, I would like to touch on a few of the many ways that alterations in developmental mechanisms may underlie the great diversity of vertebrate kidney morphologies.

When thinking about the relationships among the various vertebrate kidneys, it is helpful to consider the various types of vertebrate kidney in the context of their evolution from a postulated common ancestor as represented by the holonephric kidney. One can interpret the subsequent evolution of vertebrate kidneys as modifications of the relatively uniform holonephros. Thus, in other vertebrates, one sees specialization along the body axis, with

the posterior regions in general containing more complex kidney tissue. Selection for such specializations may be driven by animal size and/or the existence of an animal in more than one environment during its life cycle. Thus, larger animals would need larger kidneys to process the additional metabolic wastes, and such larger kidneys would most likely be generated at the posterior end of the animals, which is generated later during development. The transition to a terrestrial environment, with its strong need to conserve water, would also potentially select for a new type of kidney—the metanephros—that is more compact and that contains organizational features such as loops of Henle, which facilitate water retention. Although it is reasonable to suggest that genes that control AP patterning, such as members of the Hox family, may regulate the types of kidney tissue that develop along the AP axis, molecular data supporting this hypothesis have not yet been reported. Other genes (e.g., Eya1, Six1) that are expressed throughout the IM appear to be required only for metanephros formation, and they may be involved in regulating aspects of kidney development that are specific to the metanephros, such as the extensive proliferation that characterizes formation of the metanephros.

Another departure from the holonephros comes with respect to the role of the nephric duct during tubule differentiation. The formation of the holonephric or, more importantly, the pronephric tubules does not appear to require induction from a nephric duct. Indeed, there is evidence (in Xenopus, at least) that the pronephric tubules are patterned before the specification of the nephric duct (Brennan et al., 1998). By contrast, mesonephric and metanephric tubules require activity from the nephric duct to induce and/or promote kidney tubule differentiation. It is possible that a role for the duct in regulating tubule formation emerged after the evolution of pronephroi. Embryos with functional pronephroi typically have, for a period of development, a nephric duct that runs through a region of trunk mesoderm that does not form tubules. This situation may have led to a role for the duct in regulating the differentiation of the mesonephros and the metanephros. In this respect, it would be interesting to know whether known inducers of kidney differentiation (e.g., members of the Wnt family) are expressed in regions of the nephric duct that pass through these regions of the amphibian embryo or whether such trunk mesoderm is competent to respond to Wnt signaling before the time at which it normally differentiates into mesonephric tubules.

Comparisons among the various vertebrate kidneys also gives some insight into the development of the glomerulus. Although in the metanephroi of mammals and birds the glomerulus and the kidney tubule are intimately associated, this is almost certainly not true in the primitive vertebrate nephron. In the hagfish and many other fish and amphibian embryos, the blood-filtering site (glomerulus) and the filtrate absorption and secretion site (tubule) are separated from each other by the coelomic space. The development of more efficient nephrons, with closely associated glomeruli and tubules, had to involve a modification of developmental programs so that the tubule and the glomerulus developed from the same basic primordia. One way of thinking about this issue may be to consider the individual renal vesicles that are generated during metanephros formation as equivalent to nephrocoeles, with each renal vesicle containing tubule and glomerulus precursors. Thus, one of the modifications of the kidney developmental program that may have

occurred during vertebrate IM evolution is a movement from the formation of one large coelom in the IM to the formation of multiple coela, with each giving rise to a complete nephron unit. The molecular mechanisms by which the modification of this developmental program may have come about are not currently known.

These are but a few of the many ways in which the kidney developmental program may have been modified to produce a variety of kidney types, both within a single species and among different species. Major challenges for the future are to identify additional developmental mechanisms that regulate kidney formation and to attempt to understand how the modification of such mechanisms can generate such a wide variety of kidney forms. As reviewed previously, genetics has already contributed in a major way to the identification of the molecular pathways that regulate kidney formation, particularly in the mouse metanephros and the zebrafish pronephros. Promising areas for future research include the development of in vitro systems in which kidney development can be induced in a controlled manner and the use of genomics-based approaches to identify genes with expression that is regulated under these conditions.

SUMMARY

- The basic building block of the kidney is the nephron, which consists of a glomerulus that filters the blood, a tubule that processes the glomerular filtrate, and a duct that drains the filtrate to the outside.
- The primitive vertebrate kidney is a holonephros, which contains one nephron per body segment. Other vertebrate kidneys include the pronephros, the mesonephros, and the metanephros, all of which are modifications of the holonephric kidney.
- All kidney tissue is derived from the intermediate mesoderm (IM).
- IM formation is regulated by signals from the lateral plate (one of which is BMP) and by unknown signals from axial or paraxial tissues.
- Modifications in the kidney developmental program underlie the generation of different kidney types from the IM.
- The metanephros is the adult kidney of amniote vertebrates, and it uses specialized developmental processes to generate a highly branched organ with a large number of nephrons.
- Some developmental mechanisms that regulate metanephros formation are specialized for the formation of the metanephros, whereas others are also used in the generation of other kidney types.

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GLOSSARY

Agnathostome
A group of fish species characterized by lack of a jaw.

Amniote
The class of vertebrates that have embryos that contain amniotic membranes; this class includes mammals, birds, and reptiles. (Its opposite, the anamniote class, includes the vertebrates that lack amniotic membranes, such as fish and amphibians.)

Apodan
A class of legless amphibians that have embryos that possess holonephric-type kidneys.

Axial
Pertaining to the body axis, including the notochord and the neural tube.

Coelom (plural: coela)
The embryonic body cavity, which develops as a space within the embryonic mesoderm.

Intermediate mesoderm
A strip of mesoderm that is lateral to the somites and that gives rise to kidney tissue.

Lateral plate
Mesoderm that is lateral to the intermediate mesoderm and that gives rise to the limbs and tissues of the flank.

Nephric
Pertaining to the kidney.

Paraxial
Adjacent to the body axis; mainly pertaining to the somites.

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FURTHER READING

An excellent review of comparative kidney morphology and embryology can be found in Elizabeth Fraser's review from 1950 (Fraser, 1950). A more exhaustive treatment of the subject can be found in Goodrich's classic (Goodrich, 1930). Saxen's book-length treatment of kidney development remains the standard synthesis of the classical literature regarding kidney embryology, with an emphasis on mammalian metanephros formation (Saxen, 1987). For more contemporary treatments of kidney development, the book edited by Peter Vize (Vize et al., 2003) contains many outstanding chapters. Vize has also published an excellent review of the pronephros as a model system for studying kidney development (Vize et al., 1997). Finally, for readers interested in a compact, up-to-date review of the molecular aspects of kidney development, Dressler has recently published an excellent overview (Dressler, 2006).

36 DEVELOPMENT OF THE GENITAL SYSTEM

HONGLING DU and HUGH S. TAYLOR

Division of Reproductive Endocrinology and Infertility, Department of Obstetrics, Gynecology and Reproductive Sciences, Yale University School of Medicine, New Haven, CT

INTRODUCTION

In mammals, sex development is a genetically and hormonally controlled process, which involves three main sequential processes.

It begins with the establishment of chromosomal or genetic sex at fertilization, when a sperm (with a Y or an X chromosome) fertilizes an ovum (with an X chromosome). This initial phase of genital development represents the genetic sex determination, without any morphologic indication of sex. The early embryo is phenotypically identical in both sexes. Therefore, this is referred to as the *indifferent stage* of sexual development. Next, gonadal differentiation is initiated in accordance with the expression of sex differentiation genes. During this phase, the gonads begin to acquire sexual characteristics and differentiate into either testes or ovaries. Subsequently, the development of internal or external sexual duct systems takes place. The morphologic differentiation of sex is considered to begin with gonadal differentiation and to progress with sexual duct system development, which is influenced by the gonads.

In this chapter, we will list the genetic factors that lead to sexual and gonadal differentiation and compare them among different species. Genetic and hormonal factors, which play a role in the formation of the internal and external genitals of both sexes, will be discussed. Malformations of the genital system and its mechanisms will be described.

I. GENETIC SEX DETERMINATION

A. Sex Chromosomes

In mammals, sex determination is accomplished by a chromosomal mechanism. Whether a mammalian embryo develops into a male or a female is

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determined by its complement: XX embryos become females, and XY embryos become males. In humans, as in other mammals, females have two copies of a large X chromosome, and males have a single X and a much smaller and heterochromatic Y chromosome. The Y chromosome has a strong testis-determining effect on the indifferent stage, thus determining male gonad development. The hypothetical factor on the Y chromosome required for male differentiation is called *testis-determining factor* (TDF). The male-specific Y chromosome in mammals, in addition to playing a vital role in sex determination, also harbors the genes that are required for spermatogenesis (Tiepolo et al., 1976; Welshons et al., 1959). The number of X chromosomes appears to be unimportant to sex determination, as indicated by the loss of an X chromosome in patients with Turner syndrome (45,X or 45,XO). These patients present gonadal dysgenesis, but they are phenotypically females, with the ovaries represented by gonadal streaks.

To summarize, the presence of a Y chromosome results in the differentiation of the embryonic somatic cells of the gonad into testes rather than ovaries. The absence of a Y chromosome results in female gonad development and the formation of the ovaries. The gonads then determine the type of sexual differentiation in the internal genital ducts and the external genitalia.

Nonmammalian vertebrate species have a variety of sex chromosomal systems, such as ZZ–WZ, WX–XX–WY, and XY (Schartl, 2004). For example, birds have differentiated Z and W chromosomes in which the W is usually small and heterochromatic.

As opposed to mammals, male birds are homogametic, which means that the sex produces one type of gamete with respect to sex chromosome content. Those with two copies of the Z chromosome (ZZ) are male, whereas those with a one each of the Z and W chromosomes (WZ) are female.

In some reptiles, such as crocodilians and marine turtles, the development of the embryonic gonad into testis or ovary is dependent on temperature. By contrast, snakes have a fixed genetic sex determination system. The chromosomal system is the WZ type, and the incubation temperature of the eggs cannot influence the development of the embryonic gonads.

In amphibians, most species have homomorphic sex chromosomes, which are morphologically identical members of an homologous pair of chromosomes. However, there are also some species with heteromorphic sex chromosomes, which are a chromosome pair with some homology but that differ with regard to size, shape, and staining properties. In the Japanese frog *Rana rugosa,* populations with heteromorphic XY, homomorphic XY, and heteromorphic WZ sex chromosomes have been identified, even within the same species.

In fish, the chromosomal mechanisms show enormous variation. XY and WZ systems are the most common. In addition, XO, ZO, X_1X_2Y, XY_1Y_2, and Y autosome fusion have been described. Systems exist in which multiple sex chromosomes are present in a population. For example, three types of sex chromosomes (X, W, and Y) coexist in a population of platyfish *(Xiphophorus maculatus)*. WX, XX, and WY become females, whereas XY and YY fish become males. However, in fish and amphibians, sex can be reverted by age, social factors, and temperature or hormone treatment, or this can even occur spontaneously.

In nonmammalian vertebrates, the variety of the chromosomal system is the consequence of the dynamic process of the evolution of sex determination mechanisms.

B. The Sex Determination Gene

1. Evolution of Sex Chromosomes

In mammals, male sex determination is controlled by genes on the Y chromosome. Evolutionary comparisons show that X and Y chromosomes were originally homologous. Although the X chromosome is consistent in size and gene content, the Y chromosome is much more variable among mammalian species. A comparison of the gene content of sex chromosomes from the three major groups of extant mammals (placentals, marsupials, and monotremes) shows that part of the X chromosome and a corresponding region of the Y chromosome is shared by all mammals and thus must be very ancient. In humans, the X and Y chromosomes share two short regions at either end (the pseudoautosomal regions [PARs]) throughout which they are homologous and thus may recombine (Burgoyne, 1998). The PAR on the short arms of the X and Y chromosomes (PAR1) undergoes pairing and recombination at meiosis. By contrast, the PARs on the long arms of the X and Y chromosomes (PAR2) pair only infrequently, and the homology observed in this region is probably maintained by gene conversion. In addition, many genes and pseudogenes on the Y chromosomes have homologues on the X chromosomes. The evolution of the mammalian Y took place in several cycles of addition and attrition as autosomal regions were added to the pseudoautosomal region of one sex chromosome, recombined onto the other, and degraded on the Y.

This indicates that no matter how different in size and gene content today, the X and Y chromosomes were once homologues. The Y chromosome seems to have degraded progressively over the last 200 million years, perhaps as a consequence of keeping the sex-determining gene together with allied male-specific genes. Similar degradation occurs in single chromosomes that do not undergo recombination, irrespective of sex. Snakes have a ZZ male and a ZW female system. Like the mammalian X chromosome, the Z chromosome of snakes is large, containing about 6% of the genome in all snake families. Birds that are as distantly related as the chicken and the emu have Z chromosomes that are nearly identical genetically (Shetty, 1999). The W chromosome is considered more variable, and it has different sizes in various families of birds and snakes. The W chromosome is largely homologous to the Z chromosome in the emu, but it has become small and heterochromatic in the chicken. The process of W chromosome degradation has therefore taken place to different extents and independently in different bird and snake lineages.

The differentiation of the X and Y chromosomes is thought to have been initiated in an ancestral mammal when an allele at a single locus on the proto-Y took over a male-determining function from an ancestral genetic or environmental sex-determining system. The comparison of sequences of X-Y shared genes across species shows that the Y copy changes far more rapidly than the X copy. Comparative mapping studies show no homology between bird Z and mammalian X sex chromosomes, which indicates that the two sex chromosomes systems evolved independently. Other vertebrate classes show a wide variety of genetic and environmental sex determination mechanisms, so it is not possible to infer the sex-determining system of the common reptilian ancestor.

2. Sex-Determining Genes on the Y Chromosome in Mammals

In the mammalian XY chromosomal sex-determining system, sex differentiation depends on Y-chromosome–specific genes that trigger male development. Several testis-determining candidate genes have been identified: a minor

male-specific antigen (HYA); the zinc finger Y chromosome (ZFY); and the sex-determining region of the Y chromosome (SRY).

a. HYA

HYA is a minor male-specific antigen. It was originally discovered during the mid 1950s as a transplantation or histocompatibility antigen that caused female mice of a certain inbred strain to reject male skin of their own strain. The HYA/hya gene encoding the HYA has been mapped to the long arm of the Y chromosome in humans and the short arm of the Y chromosome in mice. During the 1970s, HYA was proposed to be the TDF as a result of two traits: HYA is uniquely expressed on male cells, and the presence of HYA is associated with testis determination as indicated by XX sex-reversed male mice. These mice have two X chromosomes, but they are clearly phenotypically male. As a result of a chromosome rearrangement, the HYA gene is expressed in these XX mice, which provides evidence that is consistent with HYA being the testis-determining Y-encoded gene (Wachtel, 1975). However, during the 1980s, it was found that HYA is absent from certain mice that develop testes and that are of indisputably male phenotype; this finding disputed the function of the HYA as the TDF (McLaren et al., 1984).

b. ZFY

Another candidate gene, *ZFY*, which is located on the Y chromosome, encodes a zinc finger protein. It lies close to the pseudoautosomal boundary on the short arm of the human Y chromosome. In the mouse, *Zfy* was found to consist of two duplicated genes, *Zfy-1* and *Zfy-2,* which are both present on the normal human Y chromosome. *ZFY* was initially considered as a TDF candidate (Page, 1987), but its lack of conservation on the Y chromosome in marsupials made it an unlikely candidate for a universal mammalian TDF. More refined mapping of the human Y chromosome defined a new minimum sex-determining region that lacked *ZFY*, and this ultimately excluded *ZFY* from a role in sex determination (Palmer et al., 1989).

c. SRY

In 1991, the *SRY* gene was cloned from a region closely linked to *ZFY*, and it has been confirmed as the TDF needed from the Y chromosome to establish male development (Figure 36.1; Koopman et al., 1990; Sinclair et al.,1990). *SRY/sry* is a small intronless gene that encodes a protein with a conserved DNA-binding high mobility group (HMG) box. *SRY* is a member of a large family of *SRY*-like HMG-box containing genes. The presence of an SRY mutation in about 15% of human XY females supported the proposition that this gene represented the TDF. The identity of *sry* as the TDF was determined in the mouse. The *sry* gene is absent in a strain of XY mice that are phenotypically female. In the transgenic mouse, *sry* can cause XX mice to undergo sex reversal and develop as males. This occurs despite the fact that they lack all other genes of the Y chromosome. *SRY* is transcribed in the genital ridges of embryos just before testis differentiation, but it is not expressed in the gonads of female mice embryos. In addition, *sry* was cloned from marsupials and shown to map to the Y chromosome, which indicates that it represents the common ancestral mammalian TDF. The *SRY* gene encodes a transcription factor that regulates the genes that are responsible for testicular development.

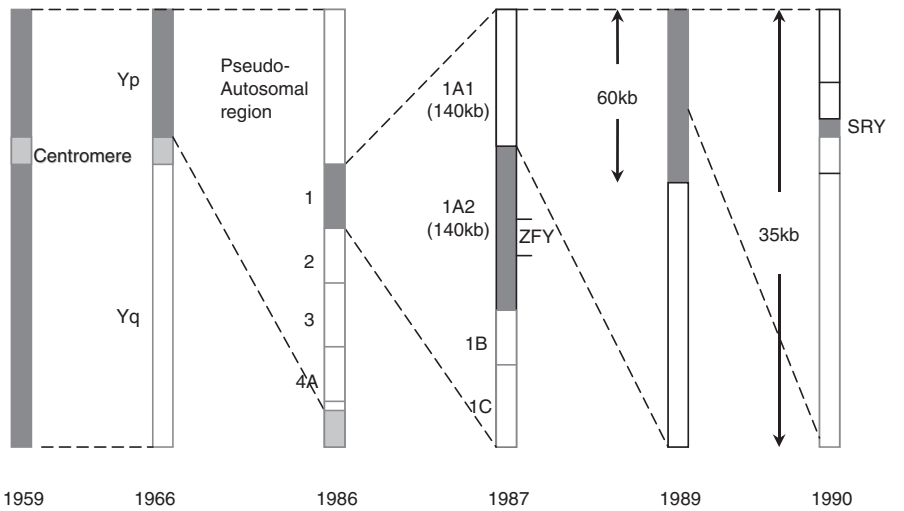

FIGURE 36.1 **A history of the sex-determination gene (*Sry*) localized on the Y chromosome.** The central role of the Y chromosome in male sex determination has been recognized for many years. In 1987, ZFY was isolated and initially equated with the TDF. In 1989, another area located near ZFY was found to play role in male sex determination. In 1991, SRY gene was isolated in this area and identified as the TDF. (Adapted from Sultan et al., 1991.)

3. Sex-Determining Genes on the Z or Y Chromosome in Nonmammalian Vertebrates

Sex-determination systems are diverse in vertebrates. As described previously, in mammals, sex differentiation depends on sex-determining genes. However, in nonmammalian vertebrates, sex is also determined by heredity, the environment, or both. Interestingly, *SRY*-like sex-determining genes have also been identified in nonmammalian vertebrates, and they are considered to be one of the sex-determining factors.

a. DMRTI

Encoding Doublesex and mab-3–related transcription factor 1 (Dmrt1) is a Z-linked candidate for the male sex-determining gene. In birds, there is no copy of Dmrt1 on the W chromosome, and males have two copies of the gene. Dmrt1 has been implicated in male sexual development in many vertebrate species, and it is considered to be the "Sry gene" of nonmammalian vertebrates (Zarkower, 2001). Recent advances in the characterization of the human Dmrt0001 gene shows that multiple transcripts are expressed in the human testis. Thus, Dmrt1 is likely to have an important role in the evolution of the sexual development mechanisms of many species (Cheng et al., 2006).

b. DMY

The DM domain gene on the Y chromosome (*DMY*) has been found in the sex-determining region of the Y chromosome of the teleost medaka fish *Oryzias latipes*. Mutations of the *DMY* cause a simple sex reversal in medaka, and this is confirmed by naturally occurring mutant females in several wild populations. *DMY* appears to be closely related to *Dmrt1* with regard to both nucleotide

sequence (93% identity) and function (Matsuda, 2003). So far, the evolutionary comparison of sex-determination genes indicates that molecular similarities among phyla are only present in the fly *doublesex,* the worm *mab-3,* and the vertebrate *Dmrt1* (dsx- and mab3-related transcription factor 1)/*Dmy* genes.

II. GONADAL DIFFERENTIATION

A. Primordial Germ Cell Migration

Primordial germ cells (PGCs) are the embryonic precursors of the gametes. In all systems, PGCs form far from the site of the developing gonads and migrate to the sites of developing ovaries or testes. This isolation may be important in the maintenance of their unique characters.

It is accepted that PGC migration occurs in three phases: separation, migration, and colonization. Several mechanisms have been hypothesized to explain PGC migration, including self-movement, attraction by chemotactic factors, PGC–PGC interactions, substrate guidance, and interaction with extracellular matrix molecules.

In human embryos, PGCs are visible early during the fourth week of gestation among the endodermal cells from the posterior wall of the yolk sac, near the origin of the allantois. During the folding of the embryo, part of the yolk sac is incorporated into the embryo, and PGCs migrate along the wall of the hindgut and through the dorsal mesentery, until they approach the newly appearing genital ridges late in the fifth week. During the sixth week, PGCs migrate into the underlying mesenchyme, and they become incorporated into the primary sex cords. The migration of PGCs is considered to be accomplished by the active ameboid movement of the cells in response to a permissive extracellular matrix substrate.

In the mouse, PGCs are induced to form in the proximal epiblast, where their formation is dependent on the expression of bone morphogenetic proteins 4 and 8b in extraembryonic tissue (Lawson et al., 1999; Ying et al. 2001). During gastrulation, they move through the primitive streak and invade the definitive endoderm, the parietal endoderm, and the allantois. In both XX and XY mice, PGCs can first be detected at 7.5 days post coitum (dpc) at the base of the allantois. By 9.0 dpc, the PGCs in the definitive endoderm become incorporated into the hindgut. Between 9.0 dpc to 9.5 dpc, PGCs migrate through the dorsal side of the hindgut to colonize the developing genital ridge. At 10.5 dpc, PGCs begin to cluster, forming a network of migrating cells. By 11.5 dpc, most PGCs have colonized the genital ridge. The entire migration takes approximately 4 days.

In mouse embryos, two genes required for the migration of PGCs have been identified: *c-kit* and *steel*. *c-kit* encodes a protein receptor that is located on the surface of the migrating PGC; *steel* encodes a growth factor that is expressed in the somatic cells that are placed in the pathway of the migrating PGC and that functions as the ligand of *c-kit*. The *steel/c-kit* interaction forms a ligand–receptor pathway that is required for PGC colonization, survival, and migration (Motro et al. 1991).

fragillis/mil-1 was recently identified as the gene that initiates PGC motility. It is a member of an interferon-inducible family of genes that is implicated in homotypic cell–cell adhesion and cell-cycle control. At 7.25 dpc, *fragilis* is

expressed in the posterior epiblast, with the highest level of expression overlapping the region where PGCs are formed. Twenty-four hours later, its expression is downregulated as PGCs scatter and move into the endoderm (Saitou et al., 2002). The expression of two other members of the fragilis gene family (*fragilis 2* and *fragilis 3*) was found in nascent PGCs that play a role in maintaining PGC migration.

PGC migration in flies can be divided into four stages: (1) internalization of the pole cells; (2) emigration of PGCs from the gut; (3) the lateral migration of PGCs; and (4) gonad coalescence.

PGCs arise from the posterior pole of the developing embryo, where localized maternal components become segregated into pole cells. Next, PGCs migrate out of the ventral side of the gut along the basal surface and into the lateral mesoderm, where they coalesce with the somatic cells of the gonad. *torso* is a maternally inherited transcript encoding a tyrosine kinase. Its activity is required for the efficient incorporation of pole cells into the hindgut pocket. *torso* signaling in flies initiates pole cell multiplication, and it may be analogous to the role of *c-kit* in PGC development in mice. The loosening of cell–cell contacts between the cells of midgut epithelium allows PGCs to emigrate from the gut, forcing them away from the gut and toward the overlying mesoderm by a repulsive signal mediated by *wunen/wunen2*. Thus, *wunen* gene expression appears to be responsible for directing the migration of the PGC in flies.

Similarly, in zebrafish, PGCs are specified by maternal components that become segregated into four clusters within the cleaving embryo. During gastrulation, these PGCs cluster more dorsally and align at the border between the head and trunk mesoderm, or they align within the lateral mesoderm. Next, PGCs migrate posteriorly to colonize the developing gonad. *dead end* is a gene that has been identified in zebrafish that plays a specific role in the initiation of PGC motility. *dead end* homologues have been identified in PGCs in Xenopus, chicken, and mouse. However, in the mouse, *dead end* is expressed in PGCs after the migratory stages; hence, its function in PGC development may not be entirely conserved.

Because there are enormous ethical, technical, and logistic problems with regard to *in vivo* studies of the movement of PGCs in humans, many experiments have been carried out in other animal models. The initiation of PGC motility is currently poorly understood, and it may be controlled by species-specific mechanisms. Despite their different origins, the early development of PGCs in flies and mice is quite similar, and the survival and early migration of PGCs in these systems require signaling via tyrosine kinase receptors (*torso* and *c-kit,* respectively). A tyrosine kinase receptor with a role similar to that of *torso/c-kit* has yet to be identified in zebrafish. The initiation of PGC motility in zebrafish is controlled by the mRNA binding protein *dead end*. PGC guidance mechanisms have been well studied in all three species, and they require chemoattractants that signal via G-protein–coupled receptors, cell–cell adhesion, and, probably, specific interactions between PGCs and the extracellular matrix.

Despite all this, evidence now suggests that there is no active migration of PGCs in the human embryo and that the displacement of germ cells can be explained by the global growth and movement of the embryo (Freeman, 2003). The analysis of recent data suggests that human PGCs do not actively migrate at any stage (either up or down) but rather that they are embedded in

the caudal tissues of the embryo (e.g., allantois, hindgut, coelomic serosa) and carried along passively during the curvilinear unrolling of this caudal region. The similarities between mouse and human embryos are so impressive that it has been doubted whether PGCs are moving independently in the mouse embryo, either. It has been proposed that, in the mammalian embryo, there is a passive carriage of multiplying cells in a caudal direction rather than an active ascent of PGCs. Such carriage of cells accompanies the normal development of the caudal part of the embryo (the so-called *embryonic unrolling*). This new hypothesis for a passive migration of PGCs in the human embryo encourages a reexamination of evidence for the previously widely accepted active migration of PGCs in other species.

B. Origin of the Gonads

The gonads are derived from three sources: the coelomic epithelium, the underlying mesenchyme, and the PGCs.

Gonadal organogenesis begins with the appearance of the genital ridge. Initially, a thickened area of coelomic epithelium develops on the medial aspect of the mesonephros. After PGCs begin to colonize the ventral region of the urogenital ridge, cells of the coelomic epithelium and the underlying mesenchymal cells undergo active proliferation. Branches of blood vessels from the dorsal aorta and the cardinal veins send endothelial cells into the genital ridges. Proliferating coelomic and mesenchymal cells, together with arriving PGCs and endothelial cells, form a cluster of condensed cells that gradually become the undifferentiated gonad.

In the mouse, the urogenital ridge develops beginning around 9.5 dpc. The morphologic establishment of the undifferentiated gonad takes approximately 48 hours (10 to 12 dpc). During this stage, the initially amorphous cluster of condensed cells becomes segregated into two compartments: (1) an epithelial compartment formed by PGCs and epithelial-like cells surrounded by a basal membrane; and (2) a stromal compartment formed by mesenchymal cells, fibroblasts, and blood vessels.

In the human, gonadal development is first identified during the fifth week of gestation. The gonads arise from an elongated region of mesoderm along the ventromedial border of the mesonephros. The indifferent gonad develops in close association with the mesonephros, an embryonic kidney that contributes to both the male and female reproductive tracts. Germ cells induce cells of the mesonephros to form the genital ridge. Cells in the cranial part of this region condense to form the adrenocortical primordia, and those of the caudal part become the genital ridges. Soon, finger-like epithelial cords called *primary sex cords* grow into the underlying mesenchyme.

C. The Bipotential Gonad and the Sex-Determining Switch

In mammals, both testis and ovary share a common origin—the bipotential gonad—that possesses neither distinctly male nor female characteristics. The indifferent gonad can differentiate into either testes or ovaries, depending on the presence or absence of a Y chromosome, respectively. The fate of the gonad is specified by the SRY gene product. In all other reproductive organs (discussed later), sexually dimorphic development does not depend directly on chromosomal compliment but rather on the presence of either the male or female gonad.

There are essentially three different cell lineages present in the gonads in addition to the germ cells. Each lineage has a bipotential fate and is capable of differentiating along either the male or female pathway.

The supporting cell lineage will give rise to Sertoli cells in the testis and follicle cells in the ovary. These cells surround the germ cells and provide an appropriate growth environment. The steroidogenic cell lineage produces the sex steroid hormones that will contribute to the development of the secondary sexual characteristics of the embryo. In the male, these correspond with the Leydig cells; in the female, these correspond with the theca cells. Finally, a connective cell lineage contributes to the formation of the organ as a whole in both the testes and the ovaries.

Early testis development is characterized by the formation of testicular cords that contain Sertoli and germ cells, with the Leydig cells excluded to the interstitium. The connective cell lineage is a major contributor to cord formation as the peritubular myoid cells surround the Sertoli cells; together, they lay down the basal lamina. The testis is also characterized by rapid and prominent vascularization.

Organization of the ovary takes place later in development and is less structured, with the connective tissue lineage giving rise to stromal cells and with no myoid cell equivalent.

There are three genes that have been identified as necessary for the development of the undifferentiated or bipotential gonad in the mouse: *wt-1, sf1,* and *lim-1* (Luo et al., 1994; Pritchard-Jones et al., 1990; Shawlot et al., 1995). The *wt-1* gene was isolated from humans who developed the Wilms' kidney tumor, and it is specifically expressed within the developing genital ridge and the kidney in mouse embryos. *wt-1* knockout mouse embryos lack kidneys and a genital ridge. The orphan nuclear receptor steroidogenic factor 1 gene (*sf1*) is a regulator of the tissue-specific expression of cytochrome P-450 steroid hydroxylases, and it is a member of the nuclear hormone receptor family. It is present in the mouse urogenital ridge at the earliest stage of organogenesis. The targeted disruption of Steroidogenic factor 1 *(sf-1)* in mice prevents the establishment of undifferentiated gonads, and all *sf-1* null mutants develop a female phenotype. *lim-1,* which is part of the LIM-homeodomain subclass of LIM proteins, is also important for urogenital development. *lim-1* null mutant mice typically die approximately 10 dpc, and the few that develop to term lack kidneys and gonads.

D. Differentiation of Testes

In mammals, testes differentiation begins at an earlier stage of development than does the differentiation of ovaries. Testis determination is normally initiated in males by the expression of the *SRY* gene on the Y chromosome in the bipotential gonad that is common to both males and females (Figure 36.2). In the human, *sry* expression can first be detected at 41 days. In the mouse, gonads, *sry* expression can be detected by 10.5 dpc, and testis differentiation occurs approximately 36 hours later, between 12.0 dpc and 12.5 dpc (Hacker et al., 1995). *sry* expression in gonadal somatic cells initiates the differentiation of Sertoli cells, which are known as the supporting cell lineage of the testis that is essential for its subsequent differentiation. Sertoli cells polarize and aggregate around germ cells to support the growth and maturation of germ cells. After *sry* expression, *sry*-related HMG box-9 *(sox9)*,

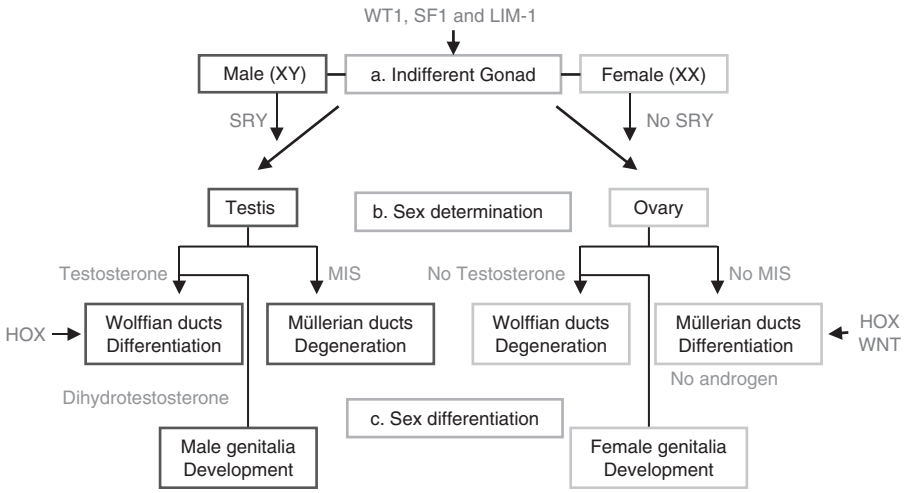

FIGURE 36.2 **Genetic and hormonal factors in the mammalian sex determination and differentiation. a,** There are three genes identified as necessary for development of the undifferentiated or bipotential gonad in the mouse: *wt1*, *sf1,* and *lim-1*. **b,** Testis determination is initiated in males by expression of the sry gene on the Y chromosome; in the absence of SRY expression, the bipotential gonad develops as an ovary. **c,** Male hormones promote both Wolffian duct and male genitalia development, while Müllerian ducts degenerate in male. In the absence of male hormones, both Müllerian ducts and female genitalia differentiate, while the Wolffian ducts degenerate in female. hox genes are involved in the development genital ducts both in male and female. The wnt gene is involved in female genital duct development.

which is another definitive testis-differentiation gene, is activated (Morais et al., 1996). *sox9* upregulation is the earliest marker of pre-Sertoli cells. *sry* and *sox9* expression overlap in Sertoli cells, colocalizing to the nucleus of pre-Sertoli cells as early as 11.5 dpc. As the *sox9*-expressing population expands between 11.5 dpc and 12.5 dpc, the number of cells that coexpress *sry* decreases until 12.5 dpc, when *sry* expression is extinguished and *sox9* expression is confined to Sertoli cells inside of the cords. Several male-specific cellular events (e.g., glycogenesis, coelomic epithelium proliferation, mesonephric migration, vasculogenesis) are induced in XY gonads after the onset of *sry* and *sox9* expression. Although the *sry* gene was discovered more than 10 years ago, the mechanism of how *sry* functions as a testis-determining factor remains unknown. Heterozygous human *sox9* mutations cause campomelic dysplasia, a severe skeletal disorder that involves defective cartilage development. Many of these male patients also have gonadal dysgenesis. Heterozygous mice that are haploinsufficient for *sox9* die perinatally as a result of skeletal malformation. *Dax1* is an X-linked orphan nuclear receptor that is expressed in the ventromedial hypothalamus, the pituitary gonadotropes, the adrenal cortex, the testis, and the ovary. The duplication of the X-chromosomal-region–spanning *Dax1* results in dosage-sensitive, male-to-female sex reversal. Alternatively, *Dax1* deficiency influences testis cord formation. It appears that appropriate *Dax1* levels are critical for normal testis development: too little or too much of the factor can have an antitestis effect. Recently, *Dax1* was reported to function as an early mediator of testis development downstream of *sry*. The analysis of $Dax1^{-/-}$ mice implies that *Dax1* functions at an early step downstream of *sry* or even possibly in a parallel path-

way of *sry* to establish Sertoli cell differentiation. The data also show that *Dax1* may play an important functional role in Leydig cells (Meeks et al., 2003).

The lifespan of fetal Leydig cells can be divided into three stages: differentiation, fetal maturity, and regression. Leydig cells differentiate after Sertoli cells during fetal development under their paracrine action. There are two signaling systems that are essential for fetal Leydig cell differentiation: the Desert hedgehog (Dhh)–Patched system and the platelet-derived growth factor (PDGF)-receptor a system (Brennan et al., 2003; Clark et al., 2000). The ligands Dhh and PDGF-A are produced by fetal Sertoli cells, whereas fetal Leydig cells express their cognate receptors (Patched for Dhh and PDGF-Ra for PDGF-A). The recognition of the involvement of these signaling systems in the process of fetal Leydig cell differentiation came from gene inactivation experiments in mice. Genetic analysis has placed PDGF-Ra upstream of Dhh. Mutants of both Dhh and PDGF-Ra have early defects in the partitioning of the testis cord and the interstitial compartment and severely impaired differentiation of fetal Leydig cells. A similar human phenotype was described for a 46,XY patient with a Dhh mutation. In both the mouse knockout and the human mutation, female external genitalia with a blind vagina were observed. Moreover, the Wolffian duct derivatives and prostate were decreased in size, indicating insufficient androgen production by fetal Leydig cells.

Anti-Müllerian hormone (AMH), which is also known as *Müllerian inhibitor substance,* is a key peptide hormone produced by Sertoli cells, and it belongs to the transforming growth factor β family. It is the earliest marker of testis formation. In the human, AMH is secreted by fetal Sertoli cells starting around 8 weeks. In the mouse, AMH transcripts are first present in the pre-Sertoli cells at 12.5 dpc, when these cells are forming cords. This factor acts as an important molecular switch to turn on a network of downstream factors that are involved in testicular development as well as male sex differentiation (Munsterberg et al., 1991).

E. Differentiation of Ovaries

In mammalian embryos, the testis pathway is the active pathway in gonad development. In mice lacking a Y chromosome (more specifically the *sry* gene), gonadal development occurs slowly. In the absence of SRY expression, the bipotential gonad develops as an ovary.

In the female mouse, the gonads retain an undifferentiated stage longer than in the male. Strings of oocytes form indistinct cords that are observed at 14 dpc. By 15 dpc, these are separated by stromal cell partitions and blood vessels. In humans, the ovary is not positively identifiable until about the tenth week of gestation. Ovarian differentiation depends on the presence of germ cells, so if primordial germ cells fail to reach the genital ridges, streak ovaries are formed. In the absence of oocytes, somatic cells transdifferentiate toward testicular tissue, and this includes the appearance of XX Sertoli cells (Nilsson et al., 2002; Nilsson et al., 2004). Similarly to the testis, the ovary contains primitive sex cords in the medullary region, but these are not as well developed as they are in the testis. In the female, the initial sex cords degenerate, but they are replaced by new epithelial proliferation that results in a new set of sex cords. These cortical sex cells superficially penetrate the mesenchyme, remaining near the outer cortex of the ovary, which is the location of the

female germ cells. Rather than forming an interconnected network, these cords form distinct clusters surrounding germ cells. This is the initial origin of the ovarian follicles. The epithelial sex cords differentiate into granulosa cells, whereas the mesenchymal cells form the thecal cells.

Relatively few genes have been shown to be specific to the early female gonadal development. *Dax1* was cloned from an X-chromosomal region in humans that was responsible for dosage-sensitive sex reversal. It is specifically expressed in XX gonads after 11.5 dpc, and it was initially suggested as a pro-ovarian or antitestis candidate gene. However, *Dax1* loss of function on the XX background does not prevent ovary development. Subsequent studies have shown an expected role for *Dax1* in testis development, which indicates that its actions are highly dependent on the timing and level of expression (Bardoni et al., 1994).

The existence of sex reversal XX individuals that develop as males in the absence of the *sry* gene led to the proposal that *sry* normally represses a factor (Z) that functions at the top of a genetic cascade as a repressor of male development. Thus, according to this theory, the Z factor would be repressed by *sry* in the male, and it would be independent of *sry* on an XX genetic background. Loss of a Z factor would be sex-reversing on XX (female-to-male). One candidate for this Z factor is Wingless-related integration site 4 (Wnt-4), which acts as a partial antitestis gene by repressing aspects of male development in the female gonad. In the mouse, Wnt genes are expressed in the mesonephric mesenchyme between 9.5 dpc and 10.5 dpc and in the gonadal mesenchyme of both sexes at 11 dpc. By 11.5 dpc, gonadal Wnt-4 expression is downregulated in the male but maintained in the female. Wnt-4 signaling is required to maintain the female germ line and to suppress the differentiation of Leydig cell precursors (McElreavey et al., 1993).

Testicular differentiation can occur in the absence of germ cells, but these cells are essential for the formation and maintenance of follicles in the ovary. In their absence, follicles degenerate into cord-like structures, and XX cells express male markers such as *sox9* and AMH (Brennan et al., 2004). In humans, a loss-of-function mutation in Wnt0004 caused Mayer–Rokitansky–Kuster–Hauser syndrome, which is characterized by the defective development of Müllerian derivatives and the duplication of a chromosomal region containing Wnt4; this condition was associated with a case of human XY sex reversal.

III. DEVELOPMENT OF THE GENITAL DUCTS

A. The Indifferent Stage

Like the gonads, the sexual ducts pass through an early indifferent stage. Unlike the gonads, in which a single tissue is bipotential, the indifferent genital ducts involve two options: the mesonephric (Wolffian) ducts and the paramesonephric (Müllerian) ducts. Both male and female embryos have two pairs of genital or sexual ducts. These ducts can differentiate into male or female reproductive organs according to the hormonal status of the fetus. In mammalian embryos, the testis secretes several hormones that promote Wolffian duct differentiation into the male reproductive tract. They form the epididymides, the ductus, and the ejaculatory system when the Müllerian ducts

degenerate. In the absence of male hormones, the Wolffian ducts degenerate, whereas the Müllerian ducts persist and differentiate into the female internal reproductive tract, which is made up of fallopian tubes, the uterus, and the superior portion of the vagina. The fate of the indifferent genital ducts depends on the gender of the gonad (Figure 36.3).

B. Development of the Male Genital Duct

Owing to the expression of SRY, the bipotential gonad of males becomes the testis. Hormones play an essential role in regulating male sexual development after the testis has formed. In mammals, this regulation depends on three key hormones produced by the fetal testis: AMH, testosterone, and insulin-like factor 3 (INSL3). In the absence of these critical testicular hormones, female sex differentiation occurs.

In males, the Müllerian duct system forms early on but subsequently regresses. The elimination of the Müllerian ducts in the male fetus is driven by AMH, which is a transforming growth factor β superfamily member (Viger et al., 2005). AMH is secreted by Sertoli cells. The expression of AMH starts around 12.5 dpc in the mouse and at around 8 weeks in the human. It is main-

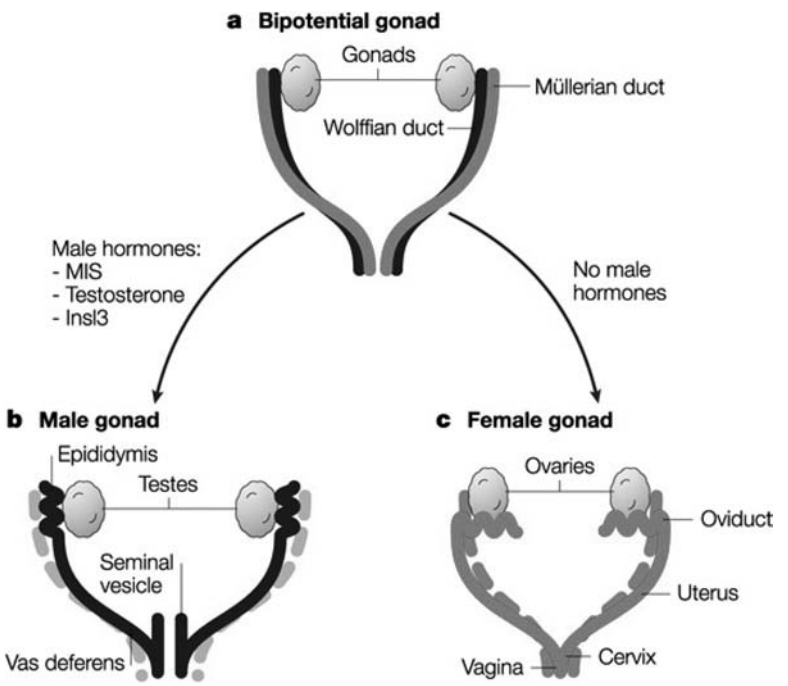

FIGURE 36.3 **Sexual differentiation of the reproductive system.** Before sexual differentiation, both male and female embryos have bipotential gonads, as they possess both Wolffian and Müllerian ducts (**a**). These ducts can differentiate into male or female reproductive organs according to the hormonal status of the fetus. Owing to the expression of *Sry*, the bipotential gonad of males becomes the testis, which secretes several hormones including testosterone, MIS, or AMH and Insl3 (**b**). Testosterone promotes Wolffian duct differentiation into the male reproductive tract, and MIS eliminates the Müllerian ducts. In females, the bipotential gonad becomes the ovary (**c**). In the absence of male hormones, the Wolffian ducts regenerate, whereas the Müllerian ducts persist and differentiate into the female reproductive tract. (Adapted from Kobayashi and Behringer, 2003.)

tained throughout fetal development, and it then declines markedly after birth. In XY embryos, AMH regulates male sex differentiation by triggering the regression of the paramesonephric (Müllerian) duct. In the human, the primary role for AMH in sex development is to cause a gradient of cranial-to-caudal regression of the Müllerian ducts during a short period from 8 to 10 weeks of gestation. This is achieved by the protein binding to a similarly expressed gradient of AMH type II receptor in mesenchymal cells, which induces apoptosis of the epithelial cells of the Müllerian ducts. In the human, the absence of AMH expression or an inactivating mutation of the AMH type II receptor gene causes persistent Müllerian duct syndrome in males. In mice, the elimination of the Müllerian duct system in male fetuses is essentially complete by 16.5 dpc. Several transcription factors are involved in the regulation of AMH expression. The first factor shown to be crucial for AMH expression is the orphan nuclear receptor *sf-1*, which was identified as an essential regulator of the sex steroid synthesis in the adrenal glands and gonads (Shen et al., 1994). In the mouse, the targeted disruption of the *sf-1* gene prevents Müllerian duct regression (Luo et al., 1994). In transgenic mice, an intact *sf-1* binding site is required for the sex-specific expression of AMH (Giuili et al., 1997). The ability of *sf-1* to activate AMH transcription has been demonstrated. This regulation is modulated through direct protein interactions with the factors Wnt1, *sox9,* and *Dax1*.

1. Androgens Are Also Essential for Normal Male Sex Differentiation

Testosterone is secreted by the Leydig cells of the testes. During fetal life, testosterone promotes virilization of the urogenital tract in two ways. First, it stimulates the mesonephric (Wolffian) ducts to develop and differentiate into the epididymides, the seminal vesicles, and the vasa deferens. Second, in the early urogenital sinus and external genitalia, testosterone is rapidly transformed into dihydrotestosterone by the enzyme steroid 5a-reductase to induce the development of the male urethra, the prostate, the penis, and the scrotum. In humans, *5a-reductase II deficiency* is a cause of pseudohermaphroditism. Affected individuals are 46,XY males who have an autosomal-recessive disorder that is characterized by an external female phenotype at birth, bilateral testes, and normally virilized Wolffian structures that terminate in the vagina.

Testosterone is also essential for testis descent into the scrotum during fetal development. Testis descent constitutes an essential step in the male sex differentiation process. In mammals, this process follows two distinct and sequential stages: the intra-abdominal stage and the inguinoscrotal stage. The INSL3 or relaxin-like factor, which is a member of the insulin-like hormone superfamily, seems to be required to regulate the intra-abdominal stage of testicular descent, although the mechanism remains poorly understood. INSL3 is expressed early in fetal mouse Leydig cells, and INSL3 knockout male mice are bilaterally cryptorchid; the gubernacular bulbs fail to develop, and they resemble normal female gubernacular structures. Evidence shows that the steroid hormones estradiol and diethylstilbestrol could downregulate INSL3 expression in the fetal Leydig cells (Nef et al., 2000).

Hox genes encode homeodomain proteins that act as transcriptional regulators. Hox genes have a well-characterized role in embryonic development, during which they determine identity along the anterior–posterior body axis (see also the chapter by Kenyon in this book). In mice and humans, Hox genes are clustered in four unlinked genomic loci (designated Hoxa-d or HOXA-D),

which contain a subset of nine to thirteen genes each. The role of mammalian Hox genes in regulating segmental patterning of axial structures and the limb is well established. Hox genes also play a similar role in the specification of developmental fate in the individual regions of the male reproductive tract. Hoxa-10 is expressed along the mesonephric duct from the caudal epididymis to the point at which the ductus deferens inserts into the urethra. Mutants of both Hoxa-10 and Hoxa-11 exhibit a homeotic transformation that results in the partial transformation of the ductus deferens to the epididymis (Bomgardner et al., 2003; Podlasek et al., 1999). Hoxa-13 and Hoxd-13 are the 5′-most members of the Hox A and D clusters. Hoxa-13 is expressed in the terminal part of the digestive and urogenital tracts during embryogenesis, specifically in the genital tubercle from which the male accessory sex organs derive (Dolle et al., 1991; Warot et al., 1997). A Hoxd-13 loss-of-function mutant demonstrated that Hoxd-13 is essential to external genital development. Hoxd-13 is the most caudally expressed Hox gene in the genitourinary tract. It is expressed in both the mesenchyme and the epithelium of the Wolffian duct and the urogenital sinus (Fromental-Ramain et al., 1996; Oefelein et al., 1996). Another homeobox gene, *emx2,* which is a mammalian homologue of the Drosophila empty spiracles, is also expressed in the epithelial component of the intermediate mesoderm. e$mx2^{-/-}$ mutants completely lack reproductive tracts and gonads. In the mutant embryos, the Wolffian duct forms on embryonic day 10.5, but it subsequently degenerates on embryonic day 11.5. *emx2* expression is only detected during the early stages of reproductive duct formation. It suggests that this gene is only required for a specific time period during the development of the intermediate mesoderm, possibly providing a survival signal.

C. Development of the Female Genital Duct

In contrast with the Wolffian duct, the Müllerian duct persists in the absence of external signals, and it must be directed to degenerate in males. In females, the absence of *sry* results in ovarian organization. In the absence of male hormones, the Wolffian ducts degenerate, whereas the Müllerian ducts persist and differentiate into the female reproductive tract, including the oviduct (fallopian tube), the uterus, and the upper portion of the vagina.

Several genes are involved in the development of female genital duct development. Although few genes have been identified in humans, mouse knockout studies have shed light on a set of genes that are essential for the regulation of Müllerian duct formation.

The Wnt gene family, which is homologous to the Drosophila Segment polarity gene Wingless, encodes secreted glycoproteins. They are involved in sex determination and the development of several female reproductive organs. In mice, a subset of Wnt gene family members has been identified to regulate Müllerian duct development.

For example, Wnt0004 expression is crucial for the formation of the Müllerian ducts, because it is required for tubule formation. Both Wnt4-deficient male and female mice completely lack Müllerian ducts, and Wnt4-mutant females even differentiate a normal male reproductive tract.

Although Wnt0004 initiates Müllerian duct formation, Wnt7a may regulate its further outcome. It is expressed in the Müllerian duct epithelium in both sexes from 12.5 dpc to 14.5 dpc. After the Müllerian duct regression, expression is lost in males. In females, it persists in the epithelium of the Mül-

lerian duct derivatives throughout life (Heikkila et al., 2001). From Wnt7a mutant female mice studies, it has been learned that Wnt7a is required for the proper differentiation of the oviduct and the uterus. In Wnt7a mutant male mice, the Müllerian ducts do not regress. This suggests that Wnt genes have a function not only in Müllerian duct formation but also in the regression of these ducts in males.

Another member of this family, Wnt5a, is also critical for female reproductive tract development; it is needed for the formation of the genital tubercle. Wnt5a-deficient female have a coiled and shortened uterus and a poorly defined cervix and vagina.

The Hox gene family has also been identified to regulate female reproductive tract development. As described previously, Hox genes play a role in male genital duct development. This chapter will now address how Hox gene expression also directs Müllerian duct differentiation and pattern formation.

In the developing Müllerian duct, a number of posterior Abdominal B (Abd B) homeobox genes were found to be expressed in partially overlapping patterns along the anterior–posterior axis. Abd B genes are expressed according to their 3′-5′ order in the Hox clusters: Hoxa-9 is expressed in the oviduct, Hoxa-10 is expressed in the developing of the uterus, Hoxa-11 is found in the primordia lower uterus and cervix, and Hoxa-13 is seen in the upper vagina (Figure 36.4; Taylor et al., 1997). The targeted mutagenesis of these genes results in region-specific defects along the female reproductive tract. Hoxa-10 deficiency causes the homeotic transformation of the anterior part of the uterus into an oviduct-like structure, and it also causes reduced fertility in females. Hoxa-13 null embryos show agenesis of the posterior portion of the Müllerian duct. When the Hoxa-11 gene is replaced by the Hoxa-13 gene, posterior homeotic transformation occurs in the female reproductive tract: the uterus, in which Hoxa-11 but not Hoxa-13 is normally expressed, becomes similar to the more posterior cervix and vagina, in which Hoxa-13 is normally expressed. It is also revealed that Wnt7a is required for the maintenance of

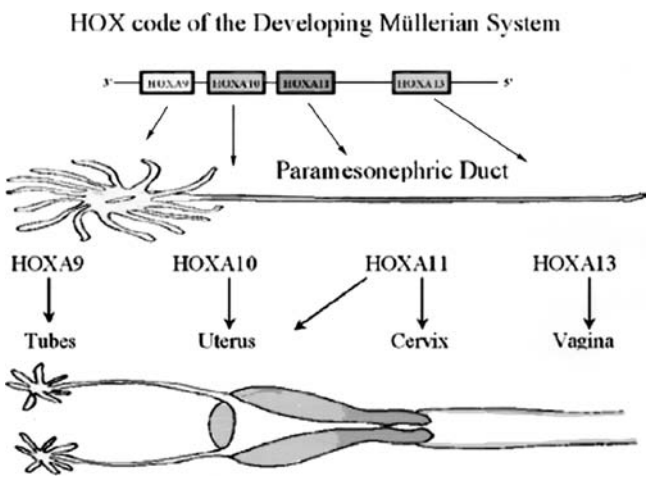

FIGURE 36.4 **Expression of HOX gene is arranged in the linear fashion along the paramesonephric duct.** Hox9 is expressed in areas destines to become fallopian tube. Hoxa10 is expressed in the developing uterus. Hoxa11 is expressed in the primordia of the lower uterine segment and cervix and upper vagina in the developing uterus and cervix. Hoxa13 is expressed in the upper vagina. (Adapted from Taylor, 2000. See color insert.)

Hoxa-10 and Hoxa-11 expression in the uterus in mice. Wnt5a resides in the same genetic pathway as Wnt7a and Hoxa genes during female reproductive tract development, and *emx2* also plays a role in female reproductive tract development. In contrast with the Wolffian duct, the Müllerian duct never forms in *emx2* mutants.

Genes such as *lim1* and Paired-box gene 2 *(pax2)* are also indispensable for the early steps of Müllerian duct development. *lim1* plays an essential role in mouse head and urogenital system development. It has a dynamic expression pattern in the Müllerian duct as early as embryonic day 11.5, which suggests that *lim1* function is crucial for the initial formation of the Müllerian duct. The analysis of *lim1* null mice revealed that Müllerian duct derivatives were completely absent. Furthermore, no Wolffian duct derivatives were ever observed in *lim1* null neonatal males, thus demonstrating that *lim1* is required for the formation of both sexual ducts. *pax2,* which is a member of the Pax gene family, encodes a homeodomain transcription factor that is homologous to the Drosophila pair-rule gene Paired. *pax2* null mice also lack a reproductive tract. Unlike the *lim1* null embryos, *pax2* null mutants present both Wolffian and Müllerian duct formation, but these subsequently degenerate. This phenotype correlates with *pax2* expression at this stage in both reproductive tracts on embryonic day 13.5, thus indicating a cell-autonomous role for *pax2* in the developing Wolffian and Müllerian ducts.

The development of the female reproductive tract also depends on estrogenic hormones by the fetal ovaries. Estrogen action is mediated by estrogen receptors (ERs), which belong to the nuclear receptor superfamily of ligand-inducible transcription factors. In fetal mice, an ERα signal was detected in the nuclei of the surrounding cells of the Müllerian ducts as early as 11.5 dpc. No expression of ERβ was detected in the mouse Müllerian duct. However, ERβs were detected in rat Müllerian ducts from 15.5 dpc to 21.5 dpc. The expression levels of ERβs are much lower than those of ERαs. The studies show that ERα is likely a dominant ER subtype that is used in Müllerian duct development (Greco et al., 1991). ERα knockout mice have a small but normally patterned reproductive tract.

IV. DEVELOPMENT OF THE EXTERNAL GENITALIA

A. The Indifferent Stage

The external genitalia also pass through an undifferentiated state before distinguishing sexual characteristics appear. The external genitalia are derived from a complex of mesodermal tissue located around the cloaca. In the human, a very early midline elevation called the *genital eminence* is situated just cephalic to the proctodeal depression. This structure soon develops into a prominent genital tubercle at the cranial end of the cloacal membrane early during the fourth week. Genitalia swellings and urogenital folds soon develop on each side of the cloacal membrane. The development of the genital tubercle is initially regulated by Hox gene expression. Located at the terminal part of the urogenital system, the genital tubercle expresses the 5′-most genes from the Hox gene clusters, specifically Hoxa-13 and Hoxd-13. The early phase of outgrowth of the genital tubercle also depends on the interacting signals of Sonic hedgehog and the fibroblast growth factors 8 and 10 (Carlson, 2004).

B. External Genitalia

The masculinization of the indifferent external genitalia is caused by androgens produced by the testis. Unlike the internal genitalia in the male, the external genitalia do not respond directly to testosterone. 5α-Reductase converts testosterone to dihydrotestosterone in the external genitalia. Under the influence of dihydrotestosterone, the genital tubercle in the male undergoes a second phase of elongation to form the penis, and the genital swellings enlarge to form the scrotal pouches. 5α-Reductase plays an important role in external genitalia development. As described previously, 5α-reductase II deficiency will cause male patients to have an external female phenotype at birth and to exhibit normally virilized Wolffian structures that terminate in the vagina.

In the absence of androgen signaling, the feminization of the indifferent external genitalia occurs. The genital tubercle in the female becomes the clitoris; the genital folds become the labia minora, and the genital swellings develop into the labia majora.

V. MALFORMATIONS OF THE GENITAL SYSTEM

Malformations of the genital system are intrinsic defects in the developing human embryo that result in localized abnormalities during the development of the reproductive duct system. Genetic events can result in congenital genital malformations during very early development stages. In the human, numerous factors can also affect the development of the reproductive tract, such as infectious agents, drugs or pharmaceutical products, environmental chemicals, physical agents, and maternal diseases. Despite their different origins, all of these factors cause some type of genetic abnormalities that ultimately induce genital malformation. As described previously, genetic abnormalities occurring in animal models, such as chromosomal anomalies and gene mutations, influence the development of the genital system, thereby causing congenital malformations.

A. Abnormalities of Sexual Differentiation

1. Turner Syndrome (Gonadal Dysgenesis)

Turner syndrome is characterized by defective gonadal development in women with a karyotypic sex chromosome abnormality (45,X or 45,XO). These individuals are phenotypic females. Individuals with this syndrome possess primordial germ cells that degenerate shortly after they reach the gonads. Affected individuals generally are of short stature, and they present with undifferentiated (streak) gonads. As expected, the internal and external reproductive structure develops as female as a result of the absence of AMH and testosterone.

2. Swyer Syndrome

Swyer syndrome, which is also known as *XY gonadal dysgenesis,* is a heterogenous condition with variant forms that are caused, in most cases, by a structural abnormality on the Y chromosome that leads to *sry* loss of function. Swyer syndrome has also been associated with autosomal mutations such as chromosome 9p deletions. Patients with Swyer syndrome are born

without functional gonads; instead, they present simply with gonadal streaks. Affected individuals are phenotypically females at birth. However, because the streak gonads are incapable of producing the sex hormones that are essential for puberty, these patients do not develop most secondary sex characteristics without hormone replacement.

3. True Hermaphroditism

Hermaphroditism is a rare condition in which ovarian and testicular tissues exist in the same person. The testicular tissue contains seminiferous tubules and spermatozoa, and the ovarian tissue contains follicles or corpora albicantia. Hermaphrodite patients typically show a chromosomal male–female mosaicism in which both the male XY and the female XX chromosome pairs are present. External genitalia may show traits from both sexes.

4. Female Pseudohermaphroditism

Female pseudohermaphroditism is characterized by male or ambiguous genitalia coupled with a female karyotype (46,XX). The XX male syndrome is a heterogeneous disorder. The presence of the *sry* gene transposed with the X chromosome leads to male differentiation. About 80% of XX males express *sry*. Some cases of *sry*-negative XX males have been reported. This may be the result of an unrecognized XX/XY chimerism or an XX/XXY mosaicism, although an autosomal-recessive disorder has also been proposed as the intrinsic cause of these less common cases.

5. Male Pseudohermaphroditism

Male pseudohermaphroditism refers to a condition that affects 46,XY individuals with differentiated testes who exhibit varying degrees of feminization. In cases of male pseudohermaphroditism, there is a spectrum of external genitalia; some individuals are completely phenotypically female, whereas others appear to be normal males with varying spermatogenesis and/or pubertal virilization. Between these two extremes is a wide area of ambiguity. Deficiency of the enzyme 5α reductase can result in external genitalia that appear to be female in an individual with an XY karyotype. High testosterone production at puberty can drive male external differentiation and an apparent "sex change" during adolescence.

6. Testicular Feminization (Androgen Insensitivity) Syndrome

Individuals with testicular feminization syndrome have a normal XY chromosomal complement; however, they are resistant to androgens (testosterone). This usually results from a mutation in the androgen receptor, which consequently leads to some extent of—or even total—external genitalia feminization. Complete testicular feminization results in an individual who looks outwardly female. Because these individuals produce AMH, the Müllerian ducts degenerate; however, the Wolffian ducts lack the ability to respond to testosterone, and they therefore regress. Patients with androgen insensitivity have no internal genitalia. The vagina in these individuals is a short structure that lacks communication with any internal organ. In these patients, breast development proceeds normally as a result of the lack of androgens; however, they still have the ability to convert testosterone to estrogen.

VI. Vestigial Structures from the Embryonic Genital Ducts

A. Mesonephric Duct Remnants

Mesonephric duct remnants are also known as *Gartner's ducts*. In females, the remains of the cranial parts of the mesonephros may persist as the epoophoron or the paroophoron. The caudal parts of the mesonephric ducts are often seen in histologic sections along the uterus or the upper vagina as Gartner's ducts. Portions of these duct remnants sometimes enlarge to form cysts.

B. Paramesonephric Duct Remnants

Remnants of the paramesonephric (Müllerian) ducts can be found in the male as a uterus-like structure. Historically, this has been called a *masculine uterus*. The remnants appear as one or two thin, uterus-like tubes that are medial to the ducti deferentes, with or without a medial corpus lying between the ampullae. The paramesonephric remnants resemble a normal female uterus, but the endometrium consists primarily of amorphous extracellular matrix.

VII. OTHER ABNORMALITIES OF THE GENITAL DUCT SYSTEM

A. Failed Müllerian Duct Fusion or Cannulation with Clinical Correlation

Normal uterine development depends on both the fusion of the two paramesonephric ducts and the absorption of the fused walls to create a single cavity. Failed Müllerian duct fusion occurs in females when the ducts do not meet or fuse. Fusion abnormalities can occur at any point along the Müllerian ducts; they may involve an isolated junction or the entire duct. The condition can have a range of results, from a small branch of the apex of the duct being affected to a complete duplication of the uterus into separate structures, each with a single fallopian tube. Failure to absorb the intervening wall between the two fused paramesonephric ducts results in a septum that separates the canals of a fused uterus. Both fusion defects and a septum can result in pregnancy complications such as miscarriage and premature delivery.

B. Congenital Absence of the Vas Deferens

The congenital bilateral absence of vas deferens occurs in males when the tubes that carry sperm from the testes (the vas deferens) fail to develop normally. This condition can occur alone or as a sign of cystic fibrosis. The testes usually develop and function normally, but sperm cannot be transported out of the epididymis. This condition occurs in men with a cystic fibrosis gene mutation; however, they often do not have any of the other health problems associated with that disease (e.g., progressive lung damage, chronic digestive system problems).

VIII. CONCLUSION

Reproductive tract development follows from an ordered set of divergent signals that begins with chromosomal complement. It is one of the few instances of such a clear bimodal heterogeneity in development. The adaptation

of conservation of these mechanisms in multiple organisms attests to their evolutionary value. The whole development includes three stages: (1) sex determination; (2) the differentiation of the internal genital ducts; and (3) the differentiation of the external genitalia. Interestingly, there are indifferent stages before the differentiation of distinguishing sexual characteristics in which numerous genetic factors are involved. The reasons that development requires this indifferent stage and the ways in which the required genes are initiated are still unclear. There are some genes, such as Hox, *lim1,* and *pax2,* that are involved in the development of the genital ducts in both males and females. How do they direct these two different structures? How is the expression of the *sry* gene initiated in the XY embryo? Finally, although the traditional dogma considers that the migration of PGCs occurs via self-movement, recent new theories question this mode of cell movement. Many questions in this field remain to be answered, and the exploitation of new genetic and genomic information will be critical in the answering of these important questions.

SUMMARY

- In mammals, sex determination is accomplished by a chromosomal mechanism. It begins at the time of fertilization through the coupling of two gametes: either two X chromosomes (XX in females) or an X and a Y chromosome (XY in males).
- The *sry* gene is the sex determination gene of the Y chromosome.
- Gonadal differentiation begins after the migration of the PGCs into the indifferent gonad. Testis determination is normally initiated in males by the expression of the *sry* gene. In the absence of *sry* expression, the bipotential gonad develops as an ovary. Ovarian differentiation is dependent on the presence of germ cells. However, germ cells are not necessary for testicular differentiation.
- The indifferent genital ducts consist of the mesonephric (Wolffian) ducts and the paramesonephric (Müllerian) ducts. In mammalian embryos, the testis secretes several hormones, such AMH, testosterone, and INSL3, that promote Wolffian duct differentiation into the male reproductive tract, whereas the Müllerian ducts degenerate in males. In the absence of the male hormones, the Wolffian ducts degenerate, whereas the Müllerian ducts persist and differentiate into the female internal reproductive tract. The fate of the indifferent genital ducts depends on the gender of the gonad.
- Genital duct development is a hormonally and genetically, controlled process. Hox gene family members are involved in the development of genital ducts in both males and females. Along the anterior–posterior axis of the genital duct, Hox genes are expressed according to their 3′-5′ order in the Hox clusters. Wnt gene family members are also involved in both female gonadal differentiation and female genital duct development.
- The external genitalia pass through an undifferentiated state before distinguishing sexual characteristics appear. The development of the genital tubercle is initially regulated by Hox gene expression. Because the genital tubercle is located at the terminal part of the urogenital system, it expresses the 5′-most genes from the Hox gene clusters, specifically Hoxa-13 and Hoxd-13.

- Malformations of the genital system are intrinsic defects in the developing human that result in localized abnormalities during the development of the reproductive duct system. The genetic abnormalities, such as chromosomal anomalies and gene mutations, influence the development of the genital system and cause malformations.

GLOSSARY

Müllerian ducts
Two embryonic tubes that extend along the mesonephros that become the uterine tubes, the uterus, and part of the vagina in the female and that form the prostatic utricle in the male. Also known as *paramesonephric ducts*.

Sex chromosome
Either of a pair of chromosomes (usually designated as *X* or *Y*) in the germ cells of most animals and some plants that combine to determine the sex and sex-linked characteristics of an individual. In mammals, XX results in a female and XY results in a male.

Sex determination
The process by which the sex of an organism is determined. In many species, the sex of an individual is dictated by the two sex chromosomes (X and Y) that it receives from its parents. In mammals, some plants, and a few insects, males are XY and females are XX; in birds, reptiles, some amphibians, and butterflies, the reverse is true. In bees and wasps, males are produced from unfertilized eggs, and females are produced from fertilized eggs. In 1991, *sry* was identified as the sex-determination gene of the Y chromosome. Environmental factors can also affect sex determination in some fish and reptiles. In turtles, for example, sex is influenced by the temperature at which the eggs develop.

Primordial germ cell
An embryonic cell that gives rise to a germ cell from which a gamete (i.e., an egg or a sperm) develops.

Wolffian duct
The duct in the embryo that drains the mesonephric tubules. It becomes the vas deferens in the male, and it forms vestigial structures in the female. Also known as the *mesonephric duct*.

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RECOMMENDED RESOURCES

The Urogenital System: http://sprojects.mmi.mcgill.ca/embryology/ug/

Human Embryology, Genital System: http://www.embryology.ch/anglais/ugenital/planmodgenital.html

Developmental Biology: 6th ed.: http://www.ncbi.nlm.nih.gov/books/bv.fcgi?rid=dbio

Recent Progress in Hormone Research: http://rphr.endojournals.org/cgi/reprint/57/1/1

Molecular Regulation of Müllerian Development by Hox Genes: http://www.annalsnyas.org/cgi/content/full/1034/1/152

Genes in Genital Malformations and Male Reproductive Health: http://www.cbra.org.br/pages/publicacoes/animalreproduction/issues/download/AR012.pdf

37

DIAPHRAGMATIC EMBRYOGENESIS AND HUMAN CONGENITAL DIAPHRAGMATIC DEFECTS

KATE G. ACKERMAN[1–3] and DAVID R. BEIER[1,2]

[1]*Division of Genetics, Brigham and Women's Hospital;*
[2]*Harvard Medical School;*
[3]*Department of Medicine, Division of Emergency Medicine, Children's Hospital, Boston, MA*

INTRODUCTION

A normal diaphragm is required for breathing and for normal pulmonary development in humans. Diaphragmatic defects are relatively common congenital disorders, and they have a significant impact on families and the health care system, because they frequently result in perinatal lethality or long-term chronic disease (West and Wilson, 2005). The most severe diaphragmatic defects result from the abnormal development of the diaphragm during early gestation. These defects, which are most commonly referred to as *congenital diaphragmatic hernias* (CDHs), are associated with abnormal pulmonary development, and they occur as frequently as 1 in every 2500 live births (Skari et al., 2000). Death or long-term complications are usually the consequences of severe respiratory insufficiency from abnormal pulmonary development and pulmonary hypoplasia. The reported mortality rate is variable, because tertiary care centers are unable to account for neonates who die before transport. Despite advances in medical therapies, population-based studies measuring outcome from the time of antenatal diagnosis report mortality rates of 58% to 79% (Beresford and Shaw, 2000; Skari et al., 2000; Stege et al., 2003). Survival is improved with an increased antenatal termination rate and with diaphragmatic defects that are not associated with other congenital anomalies (Stege et al., 2003).

The normal embryogenesis of the diaphragm is not well understood, and many different abnormal diaphragmatic phenotypes have been observed in

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humans. Although the defects associated with severe pulmonary hypoplasia usually occur in the posterior regions of the diaphragm or involve the entire hemidiaphragm, abnormalities of the anterior diaphragm are often asymptomatic and present as incidental findings in the older child or adult. Posterior diaphragmatic hernias are isolated defects in 60% of cases, whereas other affected individuals have complex CDH (i.e., they have additional malformations) or syndromic CDH (i.e., they have a constellation of anomalies that matches a syndromic pattern). Diaphragmatic defects are considered to be complex genetic disorders, and they likely occur as a result of a variety of mechanisms, including cytogenetic aberrations and *de novo* point mutations in important developmental pathway genes (Slavotinek, 2005). Heritability measured by sibling precurrence or twin studies is low, and transmission rates from affected to offspring are unknown, because low survival rates have precluded reproduction (Pober et al., 2005).

This chapter will review our current understanding of the normal and abnormal embryogenesis of the diaphragm. Until recently, the interpretation of diaphragmatic development has come from detailed anatomic evaluation in humans. During the 1980s, a teratogenic model of congenital diaphragmatic defects in rodents became widely available, which provided a valuable research tool (Costlow and Manson, 1981). Over the past 15 years, mouse mutants have provided us with more sophisticated methods of studying diaphragmatic development, and advances in genetic research in humans has allowed for candidate locus analysis and gene discovery in affected patients (Slavotinek, 2005). These complementary research strategies have begun to provide insight into the fundamental mechanisms of diaphragmatic development and its perturbation in human congenital disease.

I. DIAPHRAGMATIC ANATOMY

A. Anatomy of the Diaphragm

The diaphragm separates the thoracic cavity from the abdominal cavity, and it serves as our major respiratory muscle. Like other muscular structures, it is comprised of connective tissue, skeletal muscle, nerves, and blood vessels. The basic anatomy of the mature diaphragm is shown in Figure 37.1, A. Structures that pass through the diaphragm include the esophagus and the inferior vena cava. The esophageal orifice is surrounded by muscle, which helps to maintain the gastroesophageal junction. The muscle of the diaphragm attaches to the body wall. Anterior midline muscle is present in two distinct bundles that attach to the xiphoid process. Areas of decreased muscularization of the peripheral diaphragm occur between and lateral to these anterior muscle bundles and in the posterolateral regions, the latter regions are often called the *lumbocostal triangles*. Diaphragmatic defects may occur in any region of the diaphragm (see Figure 37.1).

B. Human Developmental Diaphragmatic Defects

Human diaphragmatic defects should be characterized by type and location. Historically, however, they have been grouped under names that comprise a variety of defects that may or may not have similar embryologic mechanisms.

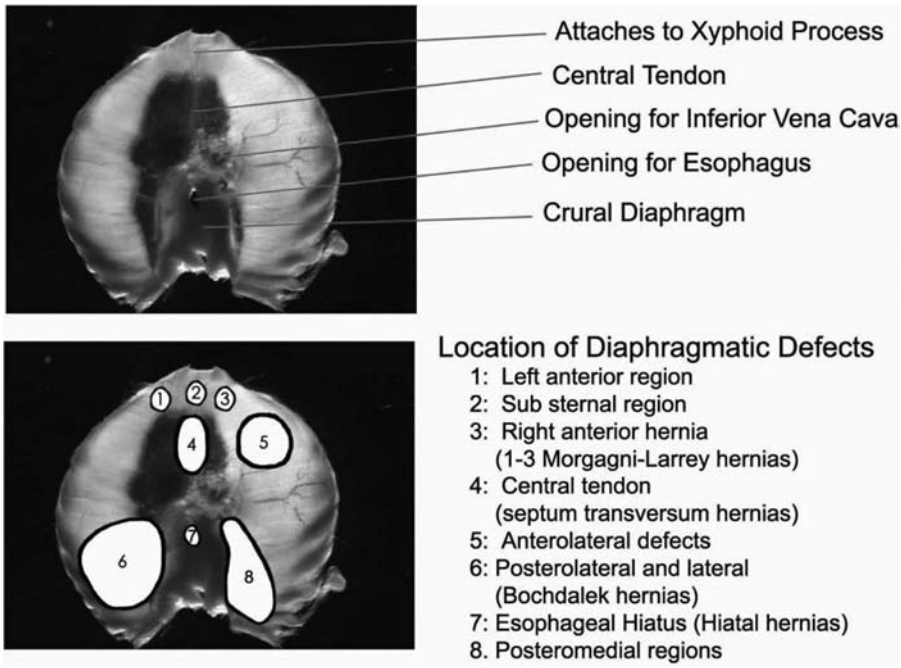

FIGURE 37.1 **Mouse diaphragms showing normal diaphragmatic structure and the location of diaphragmatic defects.** (See color insert.)

1. Type

The terminology used to describe the types of diaphragmatic defects has not been standardized. For example, the term *eventration* technically means "the upward displacement of organs into the thorax." This could arise from a lack of normal muscularization in a region, from a weakness of the connective tissue component of the diaphragm, or from a diaphragm lacking function as a result of the loss of phrenic nerve innervation. Defects may occur as a hole in the diaphragm without a membrane (i.e., hernia); they may have a thin but highly attenuated membrane (i.e., sac hernia), or they may occur as muscularization defects that cause mild herniation (i.e., eventration). The most common severe diaphragmatic defects occur in the posterior region and usually do not have a membrane or sac. Diaphragmatic defects occurring in the anterior regions almost always have a sac. The embryologic differences between these types are not understood.

2. Location

a. Posterior

Posterior or posterolateral hernias are often called *CDHs* or *Bochdalek hernias*. The name *Bochdalek hernia* was adopted after Dr. Victor Bochdalek described the posterolateral hernias in 1848 (Irish et al., 1996). Dr. Bochdalek hypothesized that these hernias occurred through the vulnerable lumbocostal triangle; this may be a mechanism for some (but not all) posterior hernias. Most human posterolateral defects are thought to arise at the site of fusion of the pleuroperitoneal fold tissue with the septum transversum tissue, causing a failure to obliterate the pleural canals or from a deficiency in pleuroperitoneal tissue,

which then fails to fuse to the septum transversum (Sweeney, 1998; Babiuk et al., 2003). Although there is limited evidence to support this presumptive mechanism, it is consistent with the examination of some defects in humans that retain posterolateral diaphragmatic tissue and that have intact lumbocostal triangles. The mechanisms of hernias in the posteromedial location are unknown. The multiple phenotypes of diaphragmatic defects in the posterior regions require careful evaluation so that a more accurate nomenclature system may be developed.

b. Anterior

Hernias occurring in the anterior regions of the diaphragm are often classified as *Morgagni hernias*. Giovanni Battista Morgagni described various types of anterior hernias during the 1700s (Irish et al., 1996). Classically, the Morgagni hernia occurs through the Morgagni foramina, which are just lateral to the anterior muscle bundles that attach to the xiphoid process. Some use the term *Morgagni hernia* to describe herniation through the right-sided foramen and the term *Larrey hernia* to refer to herniation on the left side. Others classify hernias on either side or across the midline as Morgagni–Larrey or just Morgagni hernias (Salman et al., 1999; Loong and Kocher, 2005).

c. Central or other

Hernias involving the anterior central tendon are often called *septum transversum hernias* (Paci et al., 2005), as are midline hernias that extend into the ventral wall, which are usually associated with Pentalogy of Cantrell (Wesselhoeft and DeLuca, 1984). Hernias may also occur laterally or in the anterolateral diaphragm. It is unknown how these defects should be classified, although many are large and have been associated with pulmonary hypoplasia. Defects involving the entire bilateral or hemidiaphragm usually do not involve the crural tissue. These are traditionally thought to be severe variants of the Bochdalek posterior hernia.

3. Hiatal Hernias

Hiatal hernias are generally not considered under the broad definition of CDHs, because they tend to be mild and present in older age groups, although at least some of these cases are clearly congenital. Congenital intrathoracic stomach (the most extreme case of hiatal hernia) has been reported in neonates (Hendrickson et al., 2003; Petersons et al., 2003). These defects result in the upward deviation of the stomach (often with a shortened esophagus and, in the most extreme case, an intrathoracic stomach) or in the herniation of a portion of the stomach or other abdominal contents alongside the esophagus (paraesophageal hernia). The pathogenesis of these hernias is unknown but probably heterogeneous.

4. Bilateral Defects

Bilateral diaphragmatic defects are common, especially among populations of patients who have complex CDHs with other anomalies. Bilateral defects are usually symmetric in location, but they may vary by type. For example, a patient may have a posteromedial hernia with no sac on one side and a posteromedial muscularization defect causing a mild eventration on the other. Patients with hemidiaphragmatic aplasia might have diffuse muscularization

defects on the contralateral side. Findings of different types of defects within the same patients or within members of the same family suggest that they are developmentally related (Thomas et al., 1976; Rodgers and Hawks, 1986; Elberg et al., 1989; Akel and Nasr, 2001; St. Peter et al., 2005).

II. DIAPHRAGMATIC DEVELOPMENT

A. Muscularization of the Diaphragm

Muscle must be present in the diaphragm for the normal contraction that is needed to create a negative intrathoracic pressure to inflate the lungs. Muscle is always present in a characteristic pattern that forms the main portion of the diaphragm, the diaphragmatic crus in the posterior or dorsal region, and the paraesophageal muscle (see Figure 37.1). Although it was previously believed that diaphragmatic muscle formed from an ingrowth of body wall muscle, it is now evident that this muscle is derived from separate migratory populations of muscle precursor cells. The diaphragm is a hypaxial muscular structure, just like the tongue, the limbs, the shoulder muscles, the intercostal muscles, and the abdominal muscles. Although intercostal and abdominal muscles are nonmigratory hypaxial structures, the formation of muscle in the diaphragm, tongue, shoulder, and limbs requires a complex series of signaling events to allow muscle precursor cells to delaminate from the ventrolateral somite, to maintain motility, to reach target organs, and to differentiate at the appropriate time in development (Dietrich, 1999; Birchmeier and Brohmann, 2000; Bailey et al., 2001). Most studies of this process have concentrated on limb muscle, and some of the genes required for limb muscularization are also important for other hypaxial structure muscularization (e.g., *c-Met*); however, it is clear that these processes are unique and that distinct subpopulations of muscle progenitor cells encounter and respond to different guidance cues for each structure. For example, *Lbx1* and *CXCR4/SDF-1* are important for limb and tongue muscularization, but they are not required for normal diaphragmatic muscularization (Brohmann et al., 2000; van der Weyden et al., 2002).

Genes required for normal diaphragmatic muscularization have been identified through the analysis of mutant mouse models, and they are listed in Table 37.1. *Pax3* (*paired box gene 3*) is required for the establishment of the muscle progenitor pool in the ventral dermomyotomes. Mutant *Pax3* mice (Splotch mice) have impaired development of the hypaxial muscles, and they have amuscular diaphragms (Li et al., 1999). The loss of this muscularization has been partially attributed to deficient expression of the *c-Met* receptor that is transcriptionally controlled by *Pax3* (Epstein et al., 1996). The tyrosine kinase receptor, scatter factor/hepatocyte growth factor, and its ligand, *c-Met,* control the delamination and migration of migrating muscle precursor cells (Dietrich et al., 1999). Mice deficient in *c-Met* have amuscular diaphragms (Babiuk et al., 2003). Mice with a hypomorphic mutation in the Gata transcription cofactor *Fog2 (Zfpm2)* have an abnormal pattern of diaphragmatic muscularization with the apparent overgrowth of crural muscle and a lack of posterolateral muscle. A decreased and abnormal pattern of hepatocyte growth factor expression in the region of migration onto the diaphragm was found in mutant mice (Ackerman et al., 2005). The mechanisms that guide the development of the muscle pattern in the diaphragm are unknown. The development of

TABLE 37.1 Major Mouse Models of Abnormal Diaphragmatic Muscle Migration

Gene	Phenotype
c-Met	Amuscular diaphragm[1]
Fog2 (Zfpm2)	Muscle patterning defect (hepatocyte growth factor patterning abnormal)[2]
Gab1	Amuscular diaphragm (signals c-met)[3]
MyoD	Thin diaphragm, not functional[4]
Pax3 (Splotch)	Amuscular diaphragm (signals c-met)[5]

[1]Babiuk RP, Zhang W, Clugston R, et al: Embryological origins and development of the rat diaphragm, *J Comp Neurol* 455:477–487, 2003.
[2]Ackerman KG, Herron BJ, Vargas SO, et al: Fog2 is required for normal diaphragm and lung development in mice and man, *PLoS Genet* 1:58–65, 2005.
[3]Sachs M, Brohmann H, Zechner D, et al: Essential role of Gab1 for signaling by the c-Met receptor in vivo, *J Cell Biol* 150:1375–1384, 2000.
[4]Kablar B, Krastel K, Ying C, et al: MyoD and Myf-5 differentially regulate the development of limb versus trunk skeletal muscle, *Development* 124:4729–4738, 1997.
[5]Li J, Liu KC, Jin F, et al: Transgenic rescue of congenital heart disease and spina bifida in Splotch mice, *Development* 126:2495–2503, 1999.

this characteristic patterning has been described in embryonic rats (Babiuk et al., 2003) and in embryonic muscle cell reporter mice carrying a reporter *LacZ* gene controlled by the transcription factor myocyte enhancer factor 2 (Figure 37.2; Naya et al., 1999). These studies show that the muscle is first present in the crural regions and in the central regions, where the primordial diaphragmatic tissue from the pleuroperitoneal fold start to form the diaphragm. The migratory muscle (myotubes) extend toward the lateral body walls before advancing to the anterior regions (Babiuk et al., 2003).

The phrenic nerves innervate the diaphragm. During embryogenesis, the phrenic axons initially target primordial diaphragmatic tissue in the pleuroperitoneal fold, which descends to form the posterior and lateral regions of the diaphragm. There is a close correlation between myotube formation in the diaphragm and the location and extension of phrenic intramuscular axons

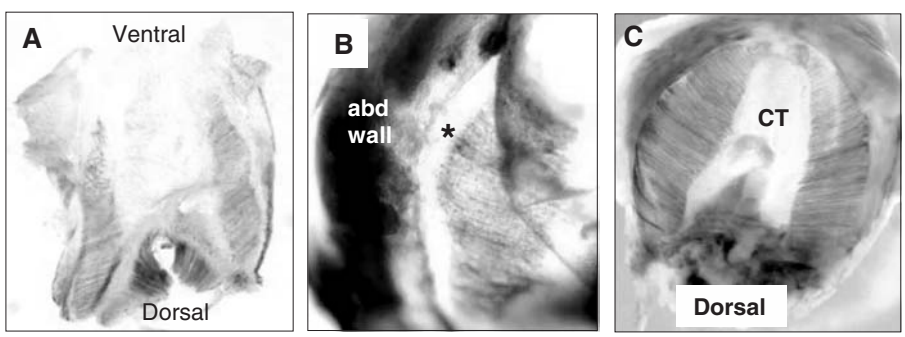

FIGURE 37.2 Early diaphragmatic muscularization in wild-type mouse embryos carrying the *desMEF2-lacZ* reporter gene, which defines embryonic muscle cells *(blue)* after X-gal staining. A and **B,** On mouse embryonic day 13.5, muscularization is present, but it has not reached the ventral regions, and it has not completely reached the abdominal wall *(*)*. **C,** On embryonic day 14.5, the muscularization is complete. Muscle never covers the central tendon region *(CT)*. (See color insert.)

with axonal growth after myotube formation (Allan and Greer, 1997; Babiuk et al., 2003). A lack of proper diaphragmatic innervation or a lack of use may result in decreased muscle cell mass or the atrophy of diaphragmatic muscle (Wolpowitz et al., 2000), and increased phrenic nerve use (through exercise such as opera singing) will cause muscle hypertrophy (Woodring and Bognar, 1998). There is no evidence that abnormal innervation of the diaphragm affects muscle cell patterning or migration.

B. Development of the Central Tendon and Connective Tissue Components

The diaphragm consists of both connective tissue and muscle, and the connective tissue portion is able to form completely if muscularization does not take place (Babiuk et al., 2003). The membranous diaphragm is thought to be derived from cells of both the septum transversum and the pleuroperitoneal folds (Greer et al., 2000; Yuan et al., 2003). The central tendon is the connective tissue portion of the diaphragm that is not populated by muscle cells (see Figure 37.1). The anterior portion of the central tendon sits under the heart, and it is attached to pericardial tissue. Severe disruptions of this region occur in humans with Pentalogy of Cantrell (Cantrell et al., 1958; Wesselhoeft and DeLuca, 1984; Carmi and Boughman, 1992). Milder disruptions are evident in mice that are deficient in the *Slit3* gene. The *Slit* family of secreted proteins is highly conserved and critical for normal embryogenesis. *Slit3* is expressed in the mesothelium of the developing diaphragm, and deficiency in two independent knockout models is associated with the herniation of thinned central tendon tissue, which fails to separate from the liver (Liu et al., 2003; Yuan et al., 2003). This has been attributed to a decrease in the proliferation of mesenchymal cells in the central tendon and to the hindered migration of mesothelial buds, which separate the liver from the diaphragm (Yuan et al., 2003). The defects observed are rare in humans, and there is no known association yet between *Slit* deficiency and abnormal human diaphragmatic development.

The majority of the membranous diaphragm is believed to be derived from cells that migrate from the pleuroperitoneal folds. The pleuroperitoneal folds are wedge-shaped structures that arise from the lateral cervical walls and fuse with the septum transversum ventrally during embryogenesis (Figure 37.3). Both migratory muscle precursor cells (expressing *Pax3* and *MyoD*) and phrenic nerves are present in the pleuroperitoneal folds (Babiuk et al., 2003). Further evidence that this tissue contributes to the diaphragm comes from the nitrofen diaphragmatic hernia model. Nitrofen (2,4-dichlorophenyl-p-nitrophenylether) is a teratogen that induces CDHs in rodent embryos after exposure in utero (Costlow and Manson, 1981). Nitrofen-induced defects occur in the posterior diaphragm and mimic the severe Bochdalek hernias seen in neonates. In rodent embryos that have been exposed to nitrofen, the structure of the pleuroperitoneal folds is abnormal, and the region of disruption corresponds anatomically with the region of absent diaphragm (Greer et al., 2000). Nitrofen also induces hernias in diaphragms that have no muscular contribution and in mice that do not grow lungs, which suggests that there is a direct effect on the integrity of the connective tissue component of the diaphragm that is not related to muscularization or lung growth (Babiuk and Greer, 2002). The mechanism of action of nitrofen is unknown, although it is at least partially dependent on retinoic acid (Greer et al., 2003).

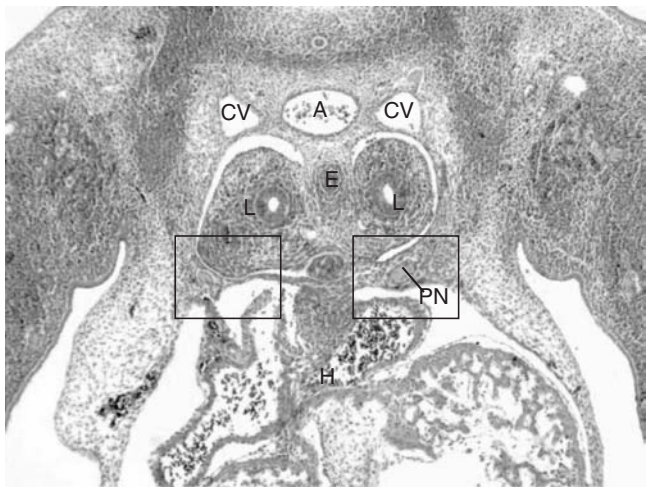

FIGURE 37.3 **Transverse section of mouse embryo on embryonic day 11.5.** The diaphragm is not yet formed at this stage. Pleuroperitoneal fold cells that are thought to contribute to the diaphragm are evident at this stage *(boxes)*. Phrenic motor axons are seen within this tissue *(PN)*. *A,* Aorta; *CV,* cardinal vein; *E,* esophagus; *L,* lung; *H,* heart.

Mice with a loss of chicken ovalbumin upstream promoter transcription factor II *(COUP-TFII,* which is also called *NrfF2)* or Wilms tumor suppressor gene *(Wt1),* also have posterior diaphragmatic membrane defects. *COUP-TFII* is a nuclear orphan receptor of the steroid/thyroid hormone receptor family (Park et al., 2003). *COUP-TFII* knockout mice die during embryogenesis before diaphragm formation, but investigators have used Cre-lox technology to create mice with a conditional loss of *COUP-TFII* in the abdominal mesenchyme, and these mice have posterior diaphragmatic defects (You et al., 2005). *Wt1* is necessary both for tumor suppression and for the normal development of many organs, including the urogenital, splenic, and cardiac systems. *Wt1* knockout mice have incomplete formation of the diaphragm (Kreidberg et al., 1993), and the PPF in *Wt1* null mice is structurally abnormal (Clugston et al., 2006). Mutations in *WT1* in humans cause a variety of defects and syndromes, some of which have been associated with congenital diaphragmatic defects (Royer-Pokora et al., 2004; Scott et al., 2005). There have been no human cases of isolated diaphragmatic defects associated with mutations in the *WT1* gene (Nordenskjold et al., 1996).

Mice with a compound loss of retinoic acid receptors also have diaphragmatic defects (Mendelsohn et al., 1994), and it is likely that the formation of the posterior diaphragm in both rodents and humans is dependent on retinoic acid. Vitamin A (retinol) and the retinoid signaling pathway are known to be crucial for normal embryogenesis (Ross et al., 2000). Retinol is transported to the cytoplasm by retinol binding proteins, where it is converted to retinoic acid before entering the nucleus to bind the retinoic acid receptors (RAR and RXR families), which leads to the regulation of many target genes (Greer et al., 2003). Diaphragmatic defects occur in rodents that are fed a diet that is deficient in vitamin A (Anderson, 1941; Wilson et al., 1953; Greer et al., 2003), and nitrofen affects embryonic retinoic acid production (Chen et al., 2003; Mey et al., 2003; Babiuk et al., 2004). In human newborns with CDH, preliminary studies have found reduced levels of both plasma retinol

and retinol-binding protein (Major et al., 1998). The CDH candidate genes *COUP-TFII, Fog2,* and *Gata4* are all suspected of playing roles in retinoic acid signaling (Malpel et al., 2000; Pereira et al., 2000; Huggins et al., 2001; Clabby et al., 2003).

III. LUNG DEVELOPMENT AND THE DIAPHRAGM

A. Pulmonary Hypoplasia Associated with Diaphragmatic Defects

Posterior diaphragmatic defects in humans are a major health problem, because they are associated with fatal or debilitating pulmonary hypoplasia. Defects in other regions of the diaphragm are usually not associated with pulmonary hypoplasia, although there are exceptions to this rule. The lungs of children with CDH have the inadequate ability to exchange gasses (likely as a result of inadequate branching and parenchymal development), and they also have pulmonary hypertension that can lead to vascular compromise. Often, these patients develop right heart failure from severe pulmonary hypertension during the newborn period. Unlike the physiologic abnormality persistent pulmonary hypertension of the newborn, the pulmonary hypertension in CDH is often only partially responsive to medical therapy. This may be the result of the severely reduced surface area of the pulmonary vascular system and of inappropriate remodeling or adaptation to elevated pulmonary pressures (Taira et al., 1998; Shehata et al., 1999; Kinsella et al., 2005; Jesudason, 2006). The mechanism of development of pulmonary hypoplasia has been a topic of considerable debate; however, it is becoming more evident that it is multifactorial and heterogeneous. Pulmonary hypoplasia may occur as a secondary defect when the lungs are compressed by herniated abdominal contents, but it also may occur as a primary defect when genes are affected that are necessary for both primary lung and primary diaphragmatic development. Those with primary lung defects are most likely those with the most severe pulmonary disease and clinical course.

B. Evidence for Secondary Pulmonary Hypoplasia

It is known that lungs develop hypoplasia and branching deficiency as a result of a lack of normal diaphragmatic excursion resulting from nervous system dysfunction (Goldstein and Reid, 1980; Fewell et al., 1981; Harding et al., 1993; Hill et al., 1994; Harding and Hooper, 1996; Harding, 1997) or abnormal muscularization (Tseng et al., 2000). When diaphragmatic hernias are created surgically in the *in utero* lamb, the animal develops pulmonary hypoplasia as a result of a compressive phenomenon (Lipsett et al., 1997; Ting et al., 1998; Lipsett et al., 2000). The contribution of secondary forces to pulmonary hypoplasia is supported by the findings of more severe abnormalities in the lung ipsilateral to the diaphragmatic defect (Areechon and Reid, 1963; Boyden, 1972; Goldstein and Reid, 1980). It has been suggested that pulmonary hypoplasia is not as severe when there are small diaphragmatic defects, because there is less herniation of abdominal contents. In general, lung size measured *in utero* correlates with respiratory outcome (Lipshutz et al., 1997; Laudy et al., 2003), although this is a subject of current controversy. Exceptions to this rule may occur as a result of technical issues or potential abnormalities in primary lung development (Heling et al., 2005).

C. Evidence for Primary Pulmonary Hypoplasia

Teratogenic (nitrofen) and genetic rodent models have provided evidence that there are common developmental mechanisms that control lung and diaphragm development. Pulmonary hypoplasia develops as a primary defect in both the nitrofen and the *Fog2* mouse models. When these lungs are removed and cultured *in vitro* (away from the influence of the developing diaphragm), they show growth or structural abnormalities. Lungs from nitrofen-exposed embryos have delayed branching, disorganization, and a reduction of late pulmonary epithelial developmental markers (Guilbert et al., 2000). Nitrofen-exposed embryos also have pulmonary smooth muscle functional abnormalities *in vitro* (Belik et al., 2003). Lungs from *Fog2* mutant mice show growth delay and a specific structural branching defect (Ackerman et al., 2005).

The CDH candidate genes *Fog2, Wt1, COUP-TFII,* and *Gata4* (Table 37.2) are all expressed in the lung during development. *Fog2* and *Gata4* are both necessary for normal structural development and growth, and they are expressed in

TABLE 37.2 Mouse Genetic Models of Congenital Diaphragmatic Hernias

Gene	Mouse Model	Human Evidence
COUP-TFII (NR2F2)	Conditional knockouts (using Nkx3.2 Cre) have posterior hernias[1]	Multiple gene deletions (syndromic)
Fog2 (Zfpm2)	Hypomorphic allele homozygotes have loss of posterolateral musculature and abnormal muscular patterning[2]	*De novo* mutation (not syndromic)
	Null allele carriers have anterior hernias, normal muscle	None
Gata4	Some null allele carriers have central hernias[3]	Multiple gene deletions
Lox	Null has central diaphragmatic rupture[4]	None
Retinoic acid receptors	Compound nulls have posterior diaphragmatic hernias[5]	None
Slit3	Null has central midline hernia[6,7]	None
Wt1	Null has posterior hernias[8]	Syndromic

[1]You LR, Takamoto N, Yu CT, et al: Mouse lacking COUP-TFII as an animal model of Bochdalek-type congenital diaphragmatic hernia, *Proc Natl Acad Sci U S A* 102:16351–16356, 2005.
[2]Ackerman KG, Herron BJ, Vargas SO, et al: Fog2 is required for normal diaphragm and lung development in mice and man, *PLoS Genet* 1:58–65, 2005.
[3]Jay PY, Bielinska M, Erlich JM, et al: Impaired mesenchymal cell function in Gata4 mutant mice leads to diaphragmatic hernias and primary lung defects. *Dev Biol* 301:602–614, 2007.
[4]Hornstra IK, Birge S, Starcher B, et al: Lysyl oxidase is required for vascular and diaphragmatic development in mice, *J Biol Chem* 278:14387–14393, 2003.
[5]Mendelsohn C, Lohnes D, Decimo D, et al: Function of the retinoic acid receptors (RARs) during development (II). Multiple abnormalities at various stages of organogenesis in RAR double mutants, *Development* 120:2749–2771, 1994.
[6]Liu J, Zhang L, Wang D, et al: Congenital diaphragmatic hernia, kidney agenesis and cardiac defects associated with Slit3-deficiency in mice, *Mech Dev* 120:1059–1070, 2003.
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[8]Kreidberg JA, Sariola H, Loring JM, et al: WT-1 is required for early kidney development, *Cell* 74:679–691, 1993.

the early developing pulmonary mesenchyme (Ackerman et al., 2006; Jay et al., 2007). *COUP-TFII* is expressed in the developing lung mesenchyme, and it probably plays a role in retinoic-acid–mediated lung development (Malpel et al., 2000). Experimental design prohibits the evaluation of lung development in the *COUP-TFII* diaphragmatic hernia mouse model, because this gene was not deleted in the lung (You et al., 2005). *Wt1* is expressed in the pulmonary mesothelium, and *Wt1* knockout mice have abnormal lungs; however, these defects have not been further characterized.

A neonate reported to have a mutation in *Fog2* had severe bilateral pulmonary hypoplasia that was out of proportion with what would have been suspected on the basis of the severity of the diaphragmatic defect (Ackerman et al., 2005). Because *Fog2* in the mouse is critical for both primary lung and diaphragm development, it is likely that this patient also had primary developmental abnormalities of both organs, which caused a severe phenotype. Hopefully, the discovery of similar associations with other candidate genes will improve our ability to better understand and predict risk on the basis of embryologic mechanisms.

IV. GENETICS OF HUMAN CONGENITAL DIAPHRAGMATIC DEFECTS/CONGENITAL DIAPHRAGMATIC HERNIA

A. Human Defects: Isolated Versus Syndromic or Complex

Human diaphragmatic defects are considered to be complex birth defects. Unlike other complex genetic diseases (e.g., asthma, diabetes) that require the inheritance and interaction of multiple genetic predisposing factors (e.g., single-nucleotide polymorphisms, mutations), diaphragmatic defects may occur (in mice) with a single identified genetic change. Because the phenotypes of these disorders are heterogeneous and the pattern of inheritance is inconsistent, it is likely that mutations or other genetic variations in many different developmental pathway genes could cause abnormal diaphragmatic development.

Diaphragmatic defects usually occur in isolation, although 30% of patients may have other anomalies (Enns et al., 1998). Nonsyndromic defects are considered to be sporadic, because the recurrence risk within a family is low (Huggins et al., 2001); however, families with multiple affected nonsyndromic diaphragmatic defects have been reported (Hitch et al., 1989; Farag et al., 1994; Kufeji and Crabbe, 1999). Because mortality and morbidity are high, good heritability data for the risk of transmission from an affected parent to an offspring are not yet available. Patients with multiple birth defects may fit the characteristic of a syndrome associated with CDH (i.e., syndromic CDH), or they may be identified as having complex CDH (i.e., not fitting a syndrome). Syndromic CDH occurs in 10% of all patients with a diaphragmatic defect, although this percentage varies widely on the basis of the population evaluated. Syndromes associated with CDH include Fryns syndrome (Slavotinek et al., 2005), Wt1 syndromes (e.g., Denys–Drash), craniofrontonasal syndrome, and Simpson–Golabi–Behmel syndrome (Slavotinek, 2005). (For a comprehensive review of syndromes associated with diaphragmatic defects, see the Congenital Diaphragmatic Hernia Overview at http://www.genetests.org.)

B. Genetic Abnormalities

Cytogenetic abnormalities determined by high-resolution G-banded karyotype probably occur in 10% of all individuals with CDH, but higher percentages have been reported for populations that have syndromic or complex cases. The most common large cytogenetic abnormalities are trisomy 18 and isochromosome 12p (Pallister–Killian syndrome). Recurrent small cytogenetic abnormalities have been reported and are a resource for the discovery of CDH candidate genes (Lopez et al., 2006). Cytogenetic CDH hot spots that are sufficiently small or common are being used for candidate gene discovery, and they are listed in Table 37.3. The effort to define small cytogenetic aberrations that

TABLE 37.3 Major Cytogenetic Hot Spots for Candidate Gene Discovery in Human Congenital Diaphragmatic Defects

Location	Evidence	Candidate Genes
1q41–42.12	Multiple deletions, one translocation (syndromic, Fryns syndrome)[1–3]	Too many to speculate
8p23.1	Multiple deletions (syndromic)[4–7]	*Gata4* most likely
8q22.3(23.1)	Multiple balanced translocations, deletion[8–10]	*Fog2*[11]
11q23.3-qter	Multiple cases with trisomy (syndromic)[12]	Too many to speculate
15q26.2	Multiple deletions (syndromic)[4,7,13]	5 Mb region, *COUP-TFII* most likely[14]

[1]Kantarci S, Casavant D, Prada C, et al: Findings from aCGH in patients with congenital diaphragmatic hernia (CDH): a possible locus for Fryns syndrome, *Am J Med Genet A* 140:17–23, 2006.
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[3]Smith SA, Martin KE, Dodd KL, Young ID: Severe microphthalmia, diaphragmatic hernia and Fallot's tetralogy associated with a chromosome 1;15 translocation, *Clin Dysmorphol* 3:287–291, 1994.
[4]Slavotinek A, Lee SS, Davis R, et al: Fryns syndrome phenotype caused by chromosome microdeletions at 15q26.2 and 8p23.1, *J Med Genet* 42:730–736, 2005.
[5]Slavotinek AM, Moshrefi A, Davis R, et al: Array comparative genomic hybridization in patients with congenital diaphragmatic hernia: mapping of four CDH-critical regions and sequencing of candidate genes at 15q26.1–15q26.2, *Eur J Hum Genet* 14:999–1008,2006.
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[8]Temple IK, Barber JC, James RS, Burge D: Diaphragmatic herniae and translocations involving 8q22 in two patients, *J Med Genet* 31:735–737, 1994.
[9]Howe DT, Kilby MD, Sirry H, et al: Structural chromosome anomalies in congenital diaphragmatic hernia, *Prenat Diagn* 16:1003–1009, 1996.
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[11]Ackerman KG, Herron BJ, Vargas SO, et al: Fog2 is required for normal diaphragm and lung development in mice and man, *PLoS Genet* 1:58–65, 2005.
[12]Klaassens M, Scott DA, van Dooren M, et al: Congenital diaphragmatic hernia associated with duplication of 11q23-qter, *Am J Med Genet A* 140:1580–1586, 2006.
[13]Klaassens M, van Dooren M, Eussen HJ, et al: Congenital diaphragmatic hernia and chromosome 15q26: determination of a candidate region by use of fluorescent in situ hybridization and array-based comparative genomic hybridization, *Am J Hum Genet* 76:877–882, 2005.

are not detectable by routine karyotypes has been advanced by array comparative genomic hybridization technology. Deletions at human chromosomes 15q26.1–26.2 have been extensively mapped to narrow the region of interest to four major candidate genes (Klaassens et al., 2005; Slavotinek et al., 2006). *COUP-TFII* is the most likely causal gene in the region based on mouse models of diaphragmatic defects (You et al., 2005). Deletions in the 8p23.1 region include the transcription factor *Gata4* (Shimokawa et al., 2005; Slavotinek et al., 2006), which interacts with *Fog2* for normal heart, lung, and probably diaphragm development (Crispino et al., 2001). Translocations in the 8q22.3–23.1 region have been reported in unrelated individuals with nonsyndromic CDH (Temple et al., 1994; Howe et al., 1996). Deletions in this region have not been found in array comparative genomic hybridization experiments, which suggests that the loss of genes in this region results in low viability. In a cohort of 30 deceased patients with diaphragmatic defects evaluated for *Fog2* mutations, one patient had a *de novo* heterozygous nonsense mutation that was predicted to result in one functional copy of *FOG2*. This child died at 5 hours of life as a result of severe respiratory distress, and was found to have a posterior diaphragmatic eventration with severe bilateral pulmonary hypoplasia. The sequencing of the DNA of patients with milder phenotypes is ongoing, but it has not revealed additional mutations. It is interesting that both *COUP-TFII* and *Gata4* interact with *Fog2* (Crispino et al., 2001; Huggins et al., 2001); this makes a developmental pathway for diaphragm development requiring these three genes likely.

VI. CONCLUSIONS

In this chapter, we have reviewed normal diaphragmatic anatomy, common diaphragmatic structural abnormalities, and the genes that are currently known to be necessary for normal diaphragmatic development. The integration of developmental genetics in animal models with human genetics and development has enhanced our current understanding of the development of the diaphragm, and the continued integration of these fields will hopefully lead us to the genetic and mechanistic classification of all human congenital diaphragmatic defects. This will be important for providing prognostic information to parents and heritability risk information to affected individuals.

SUMMARY

- Posterior hernias are associated with defects in the pleuroperitoneal folds during development.
- Defects of specific genes, including *Fog2, COUP-TF II, Gata4, Slit3,* and *Wt1,* result in defective diaphragmatic development in animal models.
- In humans, *Fog2* is the only gene that has so far been shown to be associated with isolated diaphragmatic defect. *WT1* mutations can be associated with syndromic diaphragmatic defects. *COUP-TFII* is located in a cytogenetic hot spot for CDH, and it is deleted in some humans with syndromic or complex CDH.
- Defects in pulmonary development may occur concordantly with defects in diaphragmatic development, and they may account for the poor outcomes of many affected children.

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GLOSSARY

Bochdalek hernia
A congenital diaphragmatic hernia of the posterolateral region of the diaphragm. This term is commonly used to describe any diaphragmatic hernia in the lateral or posterior region.

Central tendon (of the diaphragm)
The unmuscularized portion of the diaphragm, most of which sits under the heart and which is attached to the liver inferiorly by the falciform ligament

Congenital diaphragmatic hernia
This term is most often used to describe the Bochdalek hernia or other posterolateral diaphragmatic hernias, but it technically refers to any type of diaphragmatic hernia of congenital origin.

Eventration (of the diaphragm)
The protrusion of abdominal contents into the thoracic cavity. This term is usually used to describe a mild protrusion versus a hernia, which involves massive protrusion. Others use the term to describe a defect including the entire hemidiaphragm. An eventration may occur with or without muscularization defect.

Morgagni hernia
A diaphragmatic hernia in the anterior region of the diaphragm. This term is commonly used to describe a variety of types of anterior hernias.

Muscularization defect
A complete lack of muscle in a region of the diaphragm or abnormally patterned diaphragmatic musculature. Depending on the location and size of the muscularization defect, the result might be an eventration, a sac hernia, or no apparent protrusion of abdominal contents.

Pentalogy of Cantrell
A syndrome of congenital defects involving the abdominal wall, the sternum, the diaphragm, the pericardium, and the heart. These are considered to be ventral developmental field defects.

Pleuroperitoneal fold
Tissue in the developing embryonic thorax that will form a major part of the diaphragm and that will separate the pleural and peritoneal cavities.

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RECOMMENDED RESOURCE

Gene Tests: www.genetests.org—A publicly funded (National Institutes of Health) medical genetics information resource that includes *GeneReviews* of congenital disorders, including congenital diaphragmatic hernia.

38
FORMATION OF VERTEBRATE LIMBS

YINGZI YANG

Genetic Disease Research Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD

INTRODUCTION

There are considerable morphologic and functional differences among the limbs of different vertebrate species. Most species use their limbs to support their body weight, to walk, and to run. However, birds and bats use their forelimbs to fly, and whales use their limbs to swim. Human beings use their arms, legs, hands, and feet to perform more complicated skills and artistic tasks. However, a closer examination of all vertebrate limbs reveals that their structures are, in fact, remarkably similar. The origin of these similarities is believed to stem from the possession of a common ancestor. Most vertebrates have four limbs. No matter what purposes the limbs are serving, the skeletal elements constructing the limbs, the muscles operating them, and the nerves controlling the muscles always bear basic similarities. For example, both the mouse forelimb and the chick wing have a shoulder, a girdle, a humerus, a radius, an ulna and digits, although the number of digits varies. More importantly, the structural and functional similarities of vertebrate limbs are determined by similar developmental processes when the limbs form in the embryo.

Vertebrate limbs develop from the embryonic structure called *limb bud*. The limb bud forms by localized proliferation of the lateral plate mesoderm at certain axial levels at the dorsal–ventral (DV) boundary. In the developing mouse embryo, a visible fore limb bud appears at 9.5 days postcoitum, and the hindlimb bud forms slightly later. The limb mesenchyme gives rise to patterned limb structures that form later during development that include cartilage, bone, tendon, and ligaments. Muscle, nerves, and blood vessels are derived from cells that migrate into the limb bud during development. According to the molecular regulation and morphogenetic events, limb development can be divided into three stages: limb initiation, limb patterning, and late limb morphogenesis. This chapter will focus on the first two stages.

Principles of Developmental Genetics
Dr. Yang contributed to this article in her personal capacity. The views expressed are her own and do not necessarily represent the views of the National Institutes of Health or the United States Government.

The investigation of limb development is a very active field of developmental biology. Before the tools of molecular developmental biology were developed, classic embryological studies using mainly chick limb buds contributed significantly to the current understanding of limb development by identifying the signaling centers that control limb pattern formation. The chick limb bud has been one of the main systems of choice for embryological studies of limb development because the chick limb bud is accessible and large in size, and it can be easily manipulated *in ovo* without affecting other developmental processes. For example, signaling centers that control the three-dimensional limb patterning were identified through tissue recombination experiments in the chick limb bud, which could not have been performed in mouse embryos, which develop *in utero*. More recently, the availability of genetic tools in mice in combination with embryologic studies in chick have led to the identification of the signaling molecules and pathways that mediate that function of the previously identified signaling centers, which will be discussed further in this chapter. Because of the pleiotrophic roles of the signaling pathways identified in limb development, molecular human and mouse genetic studies have identified genetic variants in the limb signaling pathways leading to both human birth defects and diseases, such as polydactyly, brachydactyly, Greig cephalopolysyndactyly syndrome, Gorlin syndrome, and Bardet–Biedl syndrome.

I. LIMB INITIATION

The positions of limb bud formation along the anterior–posterior (AP) axis are determined genetically in embryonic development. The area in the lateral plate mesoderm that is competent to form a limb is called the *limb field*. The limb field was identified by tissue graft experiments in classic embryological studies (Kieny, 1960; 1968). In the chick embryo, when it is grafted to an ectopic location, grafted limb field tissue directs the development of an entire limb. It is interesting to note that the size of limb field is bigger than the actual size of the lateral plate that forms a limb bud during normal development. It has been suggested that Hox genes expressed at different levels along the AP axis determine where a limb bud will form (Cohn et al., 1997; Davidson et al., 1991; Izpisua-Belmonte et al., 1991). There is a narrow time window during which the grafted lateral plate mesoderm can induce the formation of an ectopic limb. Younger mesoderm requires associated somite tissue to induce limb formation, whereas older mesoderm from the already formed endogenous limb bud loses such inductive ability (Dhouailly and Kieny, 1972).

Because the early limb bud is simply a mesoderm core covered by the surface ectoderm, limb initiation requires extensive epithelial–mesenchymal interactions. However, classic embryological work has demonstrated that it is the lateral mesenchymal cells that provide the signals to initiate the process of limb development and that determine the specific identities of the limb type (arm/leg or wing/leg) and the axial levels of limb bud formation (Detwiler, 1933; Hamburger, 1938). In the pre-limb bud mesoderm, T-box transcription factors Tbx5 and Tbx4 are expressed in the future forelimb and hindlimb areas, respectively (Agarwal et al., 2003; Naiche and Papaioannou, 2003; Takeuchi et al., 2003; see Chapter 16). However, despite the highly conserved

DNA sequences and functions between Tbx5 and Tbx4, only Tbx5 is required for limb bud initiation. No forelimb bud forms in *Tbx5*$^{-/-}$ mutant embryos, but, in *Tbx4*$^{-/-}$ embryos, hindlimb bud forms and then degenerates. The function of Tbx5 is cell-autonomous as a transcription factor. However, limb initiation requires signaling from the lateral mesoderm to the overlying ectoderm. Fibroblast growth factor 10 (Fgf10), which has been found to play a critical role in limb initiation, appears to mediate the role of Tbx5 in limb bud initiation by signaling to the overlying ectoderm. First, it was found that beads soaked in recombinant FGF1, FGF2, and FGF4 can induce the formation of a complete and morphologically normal limb when implanted to the lateral plate mesoderm (Cohn et al., 1995). Second, it was found that *Fgf10* is expressed in the limb field and that *Fgf10*$^{-/-}$ mice develop without limbs. Similar to what has been observed in the *Tbx4*$^{-/-}$ mouse embryos, a tiny limb bud does form, but it quickly degenerates in the *Fgf10*$^{-/-}$ mice (Min et al., 1998; Sekine et al., 1999). Third, in the *Tbx5*$^{-/-}$ and *Tbx4*$^{-/-}$ mouse embryos, *Fgf10* expression is either never induced or weakly expressed and then quickly lost after initial expression (Naiche and Papaioannou, 2003). Fourth, FGF receptor 2 (Fgfr2) appears to mediate Fgf10 signaling in controlling limb initiation. *Fgfr2*$^{-/-}$ mutant embryos also form very small limb buds, which degenerate quickly. The expression of *Tbx5* is intact in the *Fgfr2*$^{-/-}$ embryos (Agarwal et al., 2003). Therefore, *Tbx5* and *Tbx4* act upstream of *Fgf10* during limb initiation. Another signaling pathway that plays a critical role in limb initiation is the Wnt/β-catenin signaling pathway. Central to this pathway is the stabilization of β-catenin, which is phosphorylated and degraded in the absence of Wnt signaling. When Wnt signaling is active, β-catenin phosphorylation is inhibited, and it then translocates to the nucleus where it binds lymphoid enhancer-binding factor/T-cell factors (Lef/Tcfs) to activate downstream gene expression. *Lef1* and *Tcf1* are coexpressed with *Tbx5* or *Tbx4* in the prospective and early limb mesoderm. Expression of *Lef1/Tcf1* is also lost in the prospective limb field of the *Tbx5*$^{-/-}$ embryos. However, the expression of *Tbx5* is intact in *Lef1*$^{-/-}$/*Tcf1*$^{-/-}$ double mutant embryos (Agarwal et al., 2003). The *Lef1*$^{-/-}$/*Tcf1*$^{-/-}$ double mutant embryos also form much smaller limb buds that degenerate soon after (Galceran et al., 1999).

The epithelial–mesenchymal interaction during limb initiation is likely to be mediated by the interaction between Fgf and the Wnt/β-catenin signaling. After *Tbx5* is expressed, Wnt signaling acts in concert with *Tbx5* to activate *Fgf10* expression fully in the developing limb bud. *Tbx5* alone can activate *Fgf10* expression, whereas the canonical Wnt signaling sustains high levels of *Fgf10* expression during limb initiation (Figure 38.1; Agarwal et al., 2003). *Tbx5, Fgf10,* and *Wnt3* are initially expressed in a broader area than just the presumptive limb bud. It is likely that Tbx5 establishes a limb field by activating the expression of *Fgf10* in the mesenchyme of the limb field. Because *Wnt3* is expressed in the ectoderm overlying the presumptive limb bud mesoderm, *Wnt3* and *Fgf10* may mediate the extensive ectoderm–mesenchymal interaction during early limb initiation, and they may maintain the expression of each other by forming a positive feedback loop (see Figure 38.1). This is supported by the finding that *Wnt3* is required for limb initiation (Barrow et al., 2003). It appears that Wnt3 signals to both the limb mesenchyme and the ectoderm to regulate limb initiation and elongation. It has been demonstrated that Wnt3 signals through β-catenin within the

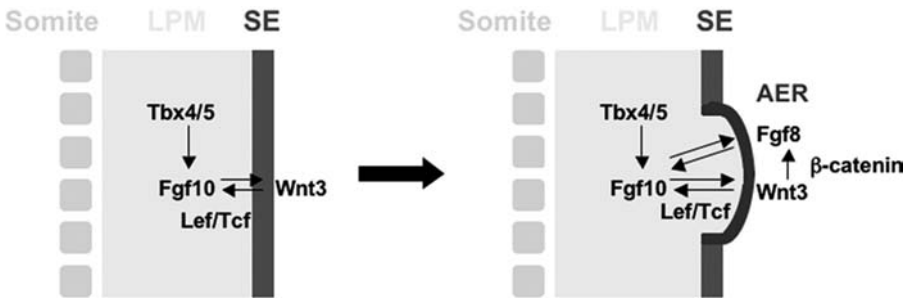

FIGURE 38.1 **Signaling pathways regulating limb initiation and apical ectoderm ridge *(AER)* formation.** *Tbx4* and *Tbx5* are expressed in the lateral plate mesoderm *(LPM)* before the expression of *Fgf10, Lef1*, and *Tcf1*. *Tbx4* and *Tbx5* directly activate *Fgf10* expression. *Fgf10* and *Wnt3* expressed in the surface ectoderm *(SE)* may form a positive feedback loop to achieve the high expression level required for limb bud outgrowth and AER induction. At this stage, Wnt3 signaling for the regulation of *Fgf10* expression is mediated by *Lef1* and *Tcf1*. Wnt3 signals in the surface ectoderm itself through β-catenin, which leads to AER induction and *Fgf8* expression. After *Fgf8* is induced, it joins the *Wnt3/Fgf* positive feedback loop by forming a positive feedback loop with *Fgf10*. This new *Fgf8–Fgf10* positive feedback loop is required for AER maintenance and proximal–distal limb outgrowth.

surface ectoderm to control the formation of the apical ectoderm ridge (AER; see Figure 38.1). AER is a thickened epithelial structure that forms at the DV boundary of the early limb bud, and it is required for limb bud outgrowth. AER formation is a critical developmental event during limb initiation. The removal of either *Wnt3* or *β-catenin* genetically from the early limb ectoderm blocks AER formation (Barrow et al., 2003), and the limb development in these mutant embryos phenocopies that seen in the $Fgf10^{-/-}$and the $Lef1^{-/-}$/ $Tcf1^{-/-}$mouse embryos. In the $Lef1^{-/-}/Tcf1^{-/-}$mouse embryos, all Wnt/β-catenin activity in the presumptive limb region may be blocked. Because early limb mesoderm as well as Fgf10 induces AER formation that can also be induced by Wnt/β-catenin signaling within the ectoderm without *Fgf10*, it appears that *Wnt3* expression is induced by the early presumptive limb mesoderm, possibly through Fgf10.

II. LIMB BUD OUTGROWTH AND PATTERNING

The limb is a three-dimensional structure with three axes: AP (thumb to little finger), proximal–distal (PD; shoulder to finger tip), and DV (back of hand to palm). Proper limb bud development requires patterning along the three axes, which is controlled by three signaling centers. The three signaling centers are established after limb initiation, and then the limb develops autonomously by the coordination among these three signaling centers.

AER is the signaling center that directs PD limb outgrowth. The function of AER in PD limb development was identified by the classic experiments performed by John Saunders in 1948 (Saunders, 1948; Summerbell, 1974). In these series of experiments, it was found that AER removal in the chick limb bud led to limb truncation along the PD axis. Earlier AER removal leads to limb truncation at more proximal limb levels. *Fgf* family members that are expressed in the AER (mainly *Fgf8* and *Fgf4*) have been identified to mediate the function of AER by molecular genetic studies in both the mouse and the

chick. In the chick limb bud, heparin beads coated with either FGF4 or FGF8 can rescue limb truncation caused by AER removal when implanted to the distal edge of the AER-stripped limb bud (Crossley et al., 1996; Niswander et al., 1993). In the mouse, the removal of *Fgf4* and *Fgf8* function in the AER blocks limb outgrowth (Sun et al., 2002).

Apart from the substantial growth of the limb bud along the PD axis, the limb bud is also patterned along the PD axis. The humerus forms at the most proximal part of the limb bud (also called the *styropod*), whereas the radius and ulna form from the middle segment of the limb bud (also called the *zeugopod*). The distal limb bud (also called the *autopod*) forms the hand plate, which includes the metatarsal bones, the tarsal bones, and the digits (Figure 38.2). For a long time, patterning along the PD axis was thought to be regulated by the progress zone (i.e., the progress zone model). In this model (Summerbell and Lewis, 1975), cells in the progress zone are progenitors of cartilage and connective tissues (see Chapter 39). Parts of the limb are specified in PD sequence by an autonomous timing mechanism operating in a "progress zone" of undifferentiated growing mesenchyme under the influence of the AER, which serves to keep the cells in the progress zone actively dividing and relatively undifferentiated (Globus and Vethamany-Globus, 1976). If the AER is surgically removed, the limb bud will stop growing, and truncation occurs along the PD axis with earlier AER removal causing more proximal limb truncation. This progress model is consistent with the order of skeletal formation in the limb, which occurs first in the more proximal limb region. A central tenet to the progress zone model is that the cells in the progress zone undergo a progressive change in positional information such that their specification depends on when they leave the progress zone and undergo differentiation. It is thought that cells that leave the progress earlier will adopt more proximal limb fates.

Recent fate mapping studies have challenged the progress zone model (Dudley et al., 2002). It is proposed in the new studies that, instead of being specified progressively as the limb bud grows out, PD specification of the limb occurs early during limb development, and patterning along the PD axis is determined at the same time. Cell-labeling experiments performed in early chick limb bud indicate that cells in the limb bud are specified as proximal or distal early on, because early-labeled limb cells were rarely found in two

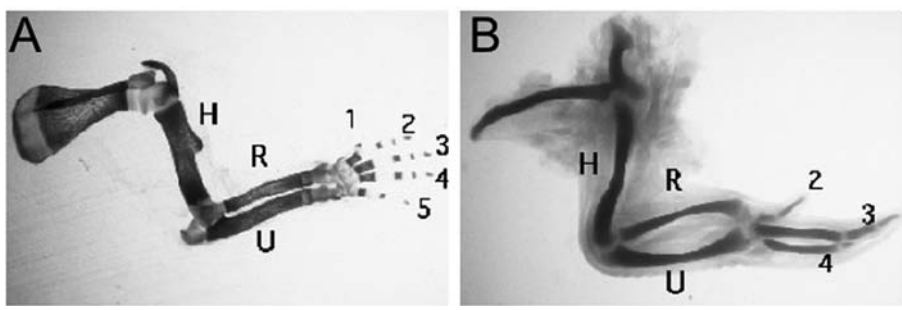

FIGURE 38.2 **Skeletal preparation of a developing forelimb in a mouse embryo *(arm, A)* and a chick embryo *(wing, B)*.** The mouse and chick limbs have similar structures. The forelimbs of the mouse and chick both contain styropod *(humerus, H)*, zeugopod (*radius [R]* and *ulna [U]*), and autopod *(digits)*. The mouse limb has five digits (1 through 5), whereas the chick wing has three digits (2 through 4; see color insert).

adjacent territories. Then, controlled by the AER, the regions of different cells expand at different times before differentiation to form the complete limb. The fate mapping study started by reassessing cell behavior in the distal chick limb bud immediately after the AER was removed. From these experiments, it is confirmed that there is extensive cell death after AER removal. Interestingly, the domain of cell loss is always the same size, regardless of when the AER is removed. Therefore, later AER removal allows a bigger part of the limb to grow. Further fate mapping studies of the presumed progress zone cells under the AER revealed that these cells died after AER removal instead of taking a more proximal fate predicted by the progress zone model. All of these studies suggest that AER prevents distal limb bud cell death, and that it is required for the expansion of prespecified limb territories along the PD axis.

This view is supported by the genetic studies of the crucial Fgf factors expressed in the AER, Fgf4 and Fgf8 (Sun et al., 2002). What was surprising is that a burst of Fgf8 in the nascent AER activated the *Fgf4* gene, which was enough to allow some skeletal elements to develop, particularly the humerus. In most cases, more distal elements also form, although both genes were inactivated completely soon after limb bud formation. Fgf signaling from the AER has two important functions. First, it is required at the earliest stages of limb development to establish a limb bud of normal size. Second, it is needed for limb bud cells to survive. The important observation in the study is that, when *Fgf4* and *Fgf8* are removed at later stages of limb development, there are not enough cells in the limb bud to produce the required skeletal elements. The *Fgf4/Fgf8* mutant limbs do not simply lose the distal limb structures.

Fgf signaling is not just required for limb outgrowth; it also plays a critical role in the PD patterning of the limb. Patterning along the PD axis of the limb is determined by the interaction of regional specific transcription factors expressed in the limb mesoderm and the signaling pathways in the AER and the limb mesoderm. Restriction of the expression of evolutionally conserved homeobox genes *Meis1* and *Meis2* to proximal regions of the limb bud is essential for limb development (Capdevila et al., 1999; Mercader et al., 2000). Ectopic *Meis2* expression in the distal limb bud severely disrupts limb outgrowth by repressing distal genes, whereas bone morphogenetic proteins (Bmps) and *Hoxd* genes restrict *Meis2* expression to the proximal limb bud. Combinations of Bmps and AER factors are sufficient to distalize proximal limb cells. Retinoic acid (RA) is an upstream activator of the proximal determinant genes *Meis1* and *Meis2*. RA promotes the proximalization of limb cells, and endogenous RA signaling is required to maintain the proximal *Meis* domain in the limb. RA synthesis and signaling range are restricted to proximal limb domains after limb initiation by the AER activity, which is mainly mediated by Fgfs. Fgfs have a specific function in promoting distalization through the inhibition of RA production and signaling.

Although the AER serves to provide the growth signal along the PD axis, the limb bud type is controlled by the mesoderm. In other words, the AER signal is permissive but not instructive for limb development. When the chick wing bud mesoderm is recombined with the leg bud ectoderm, a wing develops (Zwilling, 1955). It was also found that the development of the cross-species recombination of limbs (e.g., chick/rat) was typical of the species contributing the mesoderm (Jorquera and Pugin, 1971). Moreover, if the AP axis of the AER is reversed with reference to the mesoderm, the pattern of mesoderm differentiation is unchanged (Zwilling, 1956a). Indeed, it has been

found that *Tbx5* and *Tbx4* are expressed in the forelimb and hindlimb mesoderms, respectively, but not in the ectoderm. In addition, the hindlimb identity is determined by the mesoderm-specific transcription factor Pitx1 (Lanctot et al., 1999; Logan and Tabin, 1999; Szeto et al., 1999).

The interaction between the AER and the limb mesoderm is not one-way. Equally important is the maintenance of AER structure and function by the underlying limb mesoderm. The presumed mesoderm-derived maintenance factor is called the *apical ectoderm maintenance factor* (AEMF). In the tissue recombination experiments, it was found that an older AER would change its morphology to resemble that of a young AER if it is recombined with a young mesoderm. The normal configuration of AER is also controlled by the limb mesoderm (Zwilling, 1956b). If the limb ectoderm is recombined with non-limb mesoderm (flank lateral plate or posterior somites), then the AER degenerates within 2 days of the operation. However, if a small piece of limb mesoderm is added beneath a part of the AER, that part of the AER survives (Zwilling, 1972). It has been suggested that the AEMF is not distributed evenly through the mesoderm. Rather, it is more concentrated in the posterior half of the limb bud, which is covered by a thicker and longer AER. Because *Fgf10* forms a positive feedback loop with *Fgf8* during the limb elongation stage and this feedback loop is required for limb outgrowth and AER maintenance (see Figure 38.1), Fgf10 is obviously qualified to be an AEMF. However, Fgf10 may not be the only AEMF. A classic example of the AEMF deficiency in limb development is found in *limb deformity (ld)* mutant mice, in which AER degenerates prematurely as a result of a mesodermal defect (Kuhlman and Niswander, 1997). The molecular nature of the defective AEMF in the *ld* limb has been identified to be a secreted signaling molecule, Gremlin (Khokha et al., 2003; Zuniga et al., 1999). Gremlin is an antagonist of Bmp signaling that is expressed in the distal limb mesoderm. Because Bmp signaling promotes AER degeneration, Gremlin is required for the maintenance of AER integrity by antagonizing Bmp signaling.

The second signaling center is the *zone of polarizing activity* (ZPA), which is a group of mesenchymal cells located at the posterior limb margin and immediately adjacent to the AER. The polarizing activity was also discovered by Saunders (Saunders and Gasseling, 1968), who found that ZPA tissue grafted to the anterior limb bud leads to digit duplication in a way that is a mirror image of the endogenous digits. When limb mesoderm cells are dissociated and packed into the ectoderm jacket, the resulting limb has digits, but their specific identities cannot be determined. However, when a polarizing posterior mesenchyme graft is added, this results in a much more normal skeleton with recognizable digits, with the most posterior digit forming closest to the polarizing posterior mesenchyme graft (MacCabe et al., 1973). MacCabe has also demonstrated that there is a gradient of the polarizing posterior mesenchymal activity in the chick limb bud, and it has been found that such activity appears to be mediated by a diffusible factor (Calandra and MacCabe, 1978; MacCabe and Parker, 1976). It was proposed by Summerbell (1979) that the polarizing posterior mesenchyme emits a diffusible signal that forms a gradient and that specifies the AP axis in a dose-dependent manner.

Molecular genetic studies have identified that a vertebrate Hedgehog family member called Sonic hedgehog (Shh) mediates the activity of ZPA. Ectopically expressed *Shh* in the anterior mouse limb bud, *Shh*-expressing cells, and Shh-protein–coated beads implanted in the anterior chick limb

bud all lead to mirror-image digit duplication (Figure 38.3; Chan et al., 1995; Riddle et al., 1993). The active Shh signal corresponds to a 19-kD N-terminal peptide generated by autoproteolytic cleavage that is modified by the covalent addition of cholesterol and palmitate (Chamoun et al., 2001; Porter et al., 1995). Many studies have underscored the general long-range signaling capacity of Shh, and loss-of-function genetics has established essential Shh functions during embryogenesis, the maintenance of stem cells and disease in vertebrates. Shh has been demonstrated to act as a morphogen that patterns the DV axis of the developing neural tube by forming a morphogen gradient (Ericson et al., 1996). However, in the limb, although there is an absolute requirement of Shh signaling for AP patterning of the distal limb skeleton (because a lack of Shh in various vertebrate species results in the loss of the posterior zeugopod [ulna] and digits 5 to 2 [Chiang et al., 2001]), it is still not a settled issue whether Shh produced by the ZPA also patterns the limb bud by acting as a diffusible morphogen. Shh protein can diffuse from the ZPA to elicit a response at a distance in the limb bud mesenchyme (Lewis et al., 2001). Cells responding to Shh signaling activate the Gli1 transcription factor, but genetic analysis in the mouse shows that *Gli1* is not essential for limb bud development (Park et al., 2000). The related Gli2 protein also functions as a positive downstream mediator of Shh signaling, but, again, it is not essential for limb bud development (Mo et al., 1997). Rather, Shh signaling seems to enable the distal progression of limb bud morphogenesis and the formation of the digit arch by inhibiting the proteolytic production of the repressor form of another Gli family member, the Gli3 protein (te Welscher et al., 2002b; Wang et al., 2000), which is expressed primarily in cells that do not express *Shh*. However, when the direct contribution of ZPA cells to digit primordial was analyzed by genetic fate mapping (Harfe et al., 2004), it was found that ZPA cells give rise to descendants that either remain ZPA cells or that join a distal–anteriorly expanding population of descendants that no longer express *Shh*. Together, these two populations of Shh descendant cells give rise to the most posterior digits (5 and 4), parts of digit 3, and the ulna. Therefore, digit 2 is the only skeletal element that is not derived from cells that have expressed *Shh* at some stage. These results challenge the relevance of a spatial morphogen gradient, because digit 2 may be the only one that depends on long-range Shh signaling. Indeed, the reduction of Shh mobility across the limb bud affects digit 2, whereas digits 3 to 5 form normally. In addition, when kinetic studies were performed to address how the identities of digits 3 to 5 are specified, it was revealed that the fates of Shh descendants

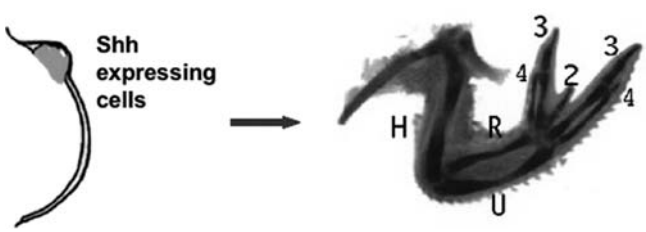

FIGURE 38.3 The implantation of *Shh*-expressing cells in the anterior limb bud leads to mirror image digit duplication. *Shh*-expressing cell pellets are implanted in the anterior chick limb bud under the apical ectoderm ridge. The ectopic Shh activity causes mirror image digit duplication (from 2–3-4 to 4–3-2–3-4; see color insert).

are progressively restricted posteriorly: descendant cells that do not express *Shh* and that are born early contribute to all three digits, whereas the ZPA cells expressing *Shh* for the longest time contribute exclusively to digit 5. It appears that limb bud cells somehow acquire a kinetic memory of the signal received. Thus, the length of time that the *Shh*-expressing cells and their non-expressing descendants that are exposed to Shh signaling earlier determines the identities of the three posterior digits. This seems to be inconsistent with the spatial morphogen gradient model, according to which long-range Shh signaling specifies digits 4, 3, and 2 (not just digit 2). However, during the dynamic process of AP limb patterning, the cells that remain in ZPA or the non-*Shh* expressing descendants closer to ZPA obviously receive higher doses of Shh. Therefore, their more posterior identity can also be explained by a spatial Shh gradient model.

It is very likely that the digit identity is patterned by both the spatial gradient and the temporal duration of the Shh morphogen. Indeed, when limb bud cells responding to Shh signaling were marked by analyzing the transcriptional activation of *Gli1* (a direct transcriptional target of Shh signaling) at specific time points of limb development, it was found that the mesenchymal cells giving rise to digits 5 to 2 and to the ulna responded to Shh signaling (Ahn and Joyner, 2004). However, although the cumulative Shh response was the highest in the posterior mesenchyme (digit 5) and progressively lower toward the anterior, no specific thresholds of response were found as predicted by the morphogen gradient model. By contrast, the Shh responsiveness of the most posterior cells (fated to form digit 5), which are exposed to Shh the longest, was reduced with time. In addition, the number of marked *Gli1*-expressing cells is reduced, and their distribution is altered in the limb buds of mouse embryos lacking the *Gli2* gene, despite the fact that *Gli2*-deficient limbs develop completely normally. These results, together with the analysis of *Gli3*-deficient limb buds, indicate that it is not the positive response to Shh as mediated by Gli1 and Gli2 but rather its inhibitory effects on Gli3 repressor (Gli3R) formation that determines digit identities. In particular, the most anterior digit 1 is specified in the absence of Shh by high levels of Gli3R, whereas cumulative high levels of Shh response effectively repress Gli3R formation and thereby specify the most posterior digit 5. Taken together, these studies indicate that the vertebrate limb bud is patterned by a kinetic memory integrating the cumulative length and strength of Shh signaling that cells receive. These temporal and spatial gradients pattern digits 2 to 5 and the ulna. These processes are regulated by controlling the inhibition of Gli3R formation at different locations along the limb AP axis and over time.

Although Shh plays a critical role in the AP patterning of the limb, the AP axis of the limb appears to be established before Shh signaling. The expression of *Shh* in the posterior limb bud is controlled by the basic helix–loop–helix transcription factor dhand. Gli3 restricts *dhand* expression to posterior mesenchyme before the activation of Shh signaling in mouse limb buds. In turn, *dhand* excludes anterior genes such as *Gli3* and *Alx4* from posterior mesenchyme. These interactions polarize the AP axis of the newly formed limb bud mesenchyme before Shh signaling (te Welscher et al., 2002a). Twist1 is also a basic helix–loop–helix transcription factor that is expressed in the developing limb bud and that is required for the maintenance of the AER. Autosomal-dominant mutations in the *twist1* gene are associated with limb and craniofacial defects in humans with Saethre–Chotzen syndrome (Jabs,

2004). The ectopic expression of *dhand* phenocopies *Twist1* loss of function in the limb, and the two factors have a gene dosage-dependent antagonistic interaction. The dimerization of Twist1 and dhand can be modulated by protein kinase A- and protein phosphatase 2A-regulated phosphorylation.

The third signaling center is the non-AER limb ectoderm that covers the limb bud. It sets up the DV polarity of not only the ectoderm but also the underlying mesoderm of the limb (reviewed by Niswander, 2002; Tickle, 2003). In the early embryo, before the limb bud forms, the DV polarity of the future limb is determined by the mesoderm as shown by ectoderm–mesoderm recombination experiments (Geduspan and MacCabe, 1989). However, before limb bud initiation, the ability to determine the DV polarity is transferred from the mesoderm to the ectoderm. If the DV polarity of the limb ectoderm is reversed relative to the mesenchyme in the early limb bud, the DV polarity of the mesenchyme changes in accordance with that of the ectoderm. At much later stages, when skeletal morphogenesis starts, the capacity of the mesoderm to respond to ectodermal control is lost.

Wnt and Bmp signaling are required to control DV limb polarity in both the limb ectoderm and the mesoderm. *Wnt7a* is expressed specifically in the dorsal limb ectoderm, and it activates the expression of *Lmx1b,* which encodes a dorsal-specific transcription factor that determines the dorsal identity (Figure 38.4; Parr et al., 1993; Riddle et al., 1995). Mutant mice with a targeted *Wnt7a* null mutation develop ventralized limbs, and the expression of *Lmx1b* is lost (Cygan et al., 1997; Parr and McMahon, 1995). On the other hand, the ectopic expression of *Wnt7a* in the ventral limb dorsalizes the limb and activates *Lmx1b* expression. During normal limb development, *Wnt7a* expression in the ventral ectoderm is suppressed by *En-1*, which encodes a transcription factor that is expressed specifically in the ventral ectoderm (see Figure 38.4; Loomis et al., 1996). In the *En-1* loss-of-function mutant limb, *Wnt7a* is ectopically expressed in the ventral ectoderm, and the limb is dorsalized. In the *En-1* and *Wnt7a* double mutant mouse, the limb is similarly ventralized like it is in the *Wnt7a* single mutant (Cygan et al., 1997; Loomis et al., 1998). These studies indicate that either ventral cell fates are default (i.e., independent of active gene regulation by *Wnt7a* and *En-1*) or that the function of *Wnt7a* is to actively suppress another ventralizing regulatory pathway.

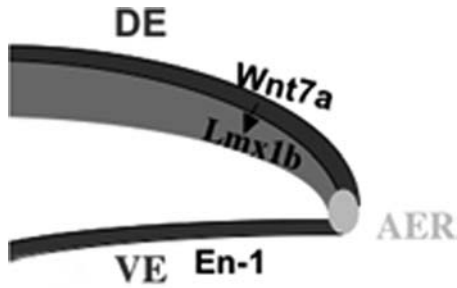

FIGURE 38.4 A schematic section of the limb bud along the dorsal–ventral axis. *Wnt7a* expressed in the dorsal ectoderm signals to the distal limb mesoderm to determine the dorsal limb identity by activating the expression of *Lmx1b*. *En-1* is expressed in the ventral ectoderm, and it is required for ventral limb development by inhibiting *Wnt7a* expression. *DE,* Dorsal ectoderm; *VE,* ventral ectoderm.

It appears that this ventralizing regulatory pathway is mediated by Bmp signaling. During early limb development in the chick, *Bmp2, Bmp4,* and *Bmp7* are expressed in the mesenchyme in an unrestricted manner along the DV axis and in the AER (Francis et al., 1994; Francis-West et al., 1995). However, in the ectoderm, these *Bmp*s are preferentially expressed in the ventral ectoderm in a way that is coincident with *En-1* and that is in a complementary pattern to that of *Wnt7a* in the dorsal ectoderm at the time the ectoderm provides DV information to the underlying mesenchyme. In the mouse limb bud, *Bmp2* is also found to be expressed in the early ventral limb ectoderm (Lyons et al., 1990). Therefore, the ventral limb mesoderm and the ectoderm receive more Bmp signaling. These Bmps signal through Bmp receptor IA *(*BMPR-IA*)* in the limb ectoderm to establish normal DV limb pattern by activating *En-1* expression in the ventral ectoderm (Ahn et al., 2001; Pizette et al., 2001). In both the mouse and the chick embryonic limb bud, the loss of Bmp signaling results in the loss of *En-1* expression and dorsalized limb, whereas activated Bmp signaling leads to ventralized limb with ectopic *En-1* expression in the dorsal ectoderm.

It appears that the effects of Bmp signaling are mediated by *Msx1* and *Msx2,* which are two transcription factors that are also themselves transcriptionally regulated by Bmp signaling. The loss of both *Msx1* and *Msx2* function in the mouse embryo also leads to the loss of *En-1* expression in the ventral ectoderm, which results in the expansion of *Wnt7a* expression, which in turn causes an expansion of *Lmx1b* expression into the ventral mesenchyme, thus leading to limb dorsalization (Lallemand et al., 2005). Therefore, the function of Bmp signaling in the early limb ectoderm is upstream of *En-1* for controlling DV limb polarity.

It is interesting to note that, like Bmp signaling, the Wnt/β-catenin signaling pathways also act directly in the limb ectoderm to control DV patterning by controlling the expression of *En-1* (Barrow et al., 2003). In contrast with *Wnt7a*, which is a dorsalizing factor, it appears that *Wnt3,* which is expressed throughout the early limb ectoderm, signals through β-catenin to ventralize the limb. Wnt3/β-catenin signaling is required in the ventral ectoderm for the expression of *En-1*. A loss of either *Wnt3* or *β-catenin* in the ventral limb ectoderm resulted in a dorsalized limb that can be attributed to loss of *En-1* expression. However, it appears that Bmps and Wnt/β-catenin also signal to the limb mesoderm directly to control DV patterning. When BmpR-IA is specifically inactivated only in the mouse limb bud mesoderm, the distal limb is also dorsalized without altering the expression of *Wnt7a* and *En-1* in the limb ectoderm (Ovchinnikov et al., 2006). Likewise, in the mouse limb bud mesoderm, the loss of *β-catenin* (like the loss of *Wnt7a* in the dorsal ectoderm) leads to limb ventralization, whereas activated β-catenin, like *Wnt7a* ectopic expression, causes limb dorsalization. Because Wnt7a signals to the limb mesoderm directly to control *Lmx1b* expression and DV limb patterning, it is possible that Wnt7a signals through β-catenin in the limb mesoderm and the Wnt/β-catenin and Bmp signaling pathways antagonize each other in controlling the expression of *Lmx1b* directly in the mesoderm.

The three signaling centers that control growth and patterning along the three axes are not functionally independent. The interaction of these three signaling centers ensures that three-dimensional growth and patterning are coordinated during vertebrate limb development (Figure 38.5). It has been shown before that polarizing posterior mesenchyme must be grafted under the AER

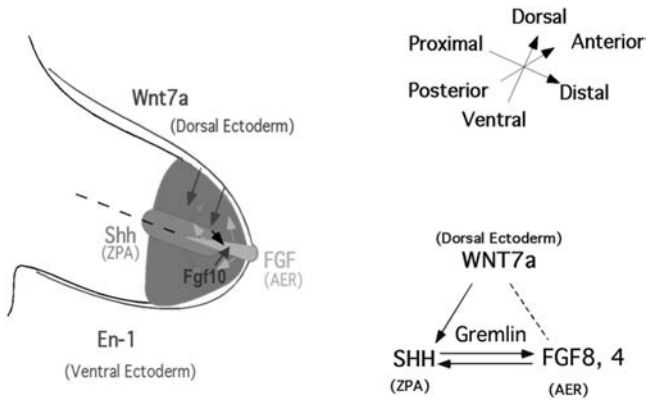

FIGURE 38.5 Patterning along the three limb axes is coordinately regulated by interactions among the signaling pathways. Both dorsal–ventral (Wnt7a) and proximal–distal (Fgfs) signals regulate anterior–posterior limb patterning by controlling *Shh* expression. The anterior–posterior signal Shh is also required for proximal–distal limb outgrowth by regulating the expression of *Fgfs* in the apical ectoderm ridge through *Gremlin*. (See color insert.)

in the anterior limb to cause mirror image digit duplication (Tickle et al., 1975); this highlights an interaction between the AP and PD signaling centers. It has also been demonstrated that the disruption of the dorsal ectoderm, which controls DV patterning, results in the shortening of limb skeletal elements along the PD axis (Martin and Lewis, 1986). This suggests that the DV signaling center also interacts with the PD signaling center. At the molecular level, it is now clear that the three signaling centers indeed interact with each other through interactions of the mediating signaling molecules (see Figure 38.5). First, there is a positive feedback loop between *Shh* and the *Fgf*s expressed in the AER, which connects AP limb patterning with PD limb outgrowth. Fgf signaling from the AER is required for *Shh* expression, and Shh signals through *Gremlin* to maintain the normal expression of *Fgf*s in the AER (Khokha et al., 2003; Laufer et al., 1994; Niswander et al., 1994). Second, the dorsalizing signal Wnt7a is required for maintaining the expression of *Shh* that patterns the AP axis (Parr and McMahon, 1995; Yang and Niswander, 1995).

III. LIMB DEVELOPMENT AND DISEASES

The three-dimensional limb development is orchestrated by the proper regulation of cell signaling and transcriptional networks. Molecular genetic studies of limb development in model organisms (mainly the mouse and the chick) have provided significant insight in both mutation identification and pathological mechanisms of human congenital limb deformities. In many cases, because limb deformities are not associated with other more detrimental defects, there has accumulated a wealth of clinical descriptions of different limb abnormalities. Because most of the signaling pathways controlling limb development also play roles in other developing processes, identifying the molecular nature of limb deformities has also enhanced our understanding of congenital diseases affecting other systems, such as the kidney and the central nervous system.

Similar to what has been shown in mouse genetic studies, mutations in the WNT, FGF, and SHH signaling pathways in humans also affect limb formation, outgrowth, and patterning along the three axes. *Wnt3* is required for limb initiation in mouse. In humans, a nonsense mutation that truncates WNT3 protein at its amino terminus has been found to be a very likely cause of the rare human genetic disorder tetra-amelia (Online Mendelian Inheritance in Man [OMIM] #273395) in four affected fetuses of a consanguineous Turkish family (Niemann et al., 2004). Tetra-amelia is characterized by the complete absence of all four limbs and other anomalies in craniofacial and urogenital development.

FGF signaling plays multiple roles in limb development. The significance of FGF signaling in the human developing limb is highlighted by the findings that mutations in FGF receptors lead to limb and skeletal deformities in Apert syndrome (AS; OMIM# 101200), Jackson–Weiss syndrome (JWS; OMIM# 123150), and Pfeiffer syndrome (PS; OMIM#101600; reviewed by Naski and Ornitz, 1998; Ornitz and Marie, 2002). AS and JWS result from mutations in FGFR2. These syndromes are characterized by syndactyly of the hands and feet and the premature fusion of the cranial sutures. PS results from a single mutation in FGFR1 or one of several mutations in FGFR2. This syndrome is characterized by broad great toes and thumbs in addition to the premature fusion of cranial sutures. Mechanistic studies of the FGFR mutations in these syndromes further indicate that some and perhaps all of the mutations causing JWS and PS are the result of activating mutations in an FGFR. In addition, it has been found that the S252W mutation of AS alters the ligand-binding affinity of FGFR2 (Yu et al., 2000). The increased affinity for FGF ligands caused by this mutation may result in the enhanced activation of the receptor in the mesenchymal cells that are destined to form the digits.

The SHH signaling pathway controls AP limb patterning. One of the most frequently observed human limb malformations is preaxial polydactyly (PPD), which involves disrupted AP limb patterning. Patients with PPD (particularly triphalangeal thumb polysyndactyly; OMIM#190605) and preaxial polydactyly type II (OMIM#174500) have extra digits on the sides of their thumbs or great toes, just like what has been observed in mouse mutants with ectopic *Shh* expression in the anterior limb bud. From the insight gained from the molecular genetic studies of the Sasquatch (Sharpe et al., 1999) and Hemimelic extratoe (Clark et al., 2000) mouse mutants with preaxial supernumerary digits, it is found that point mutations in the long-range, limb-specific regulatory element of the human SHH gene are responsible for the human limb abnormality of PPD (Lettice et al., 2002).

GLI3 transduces the Hedgehog signal, and mutations in *GLI3* result in Greig cephalopolysyndactyly syndrome (OMIM# 175700) or Pallister–Hall syndrome (OMIM# 146510). Mutations such as deletions or translocations resulting in the haploinsufficiency of GLI3 are found in association with Greig cephalopolysyndactyly syndrome (Brueton et al., 1988; Pettigrew et al., 1991; Vortkamp et al., 1991), whereas mutations resulting in dominant-negative GLI3 are found in Pallister–Hall syndrome (Johnston et al., 2005; Kang et al., 1997). In addition, a mutation at codon 764 of the *GLI3* gene, which is three-prime to the conserved domain called post zinc finger-1, is found to cause postaxial polydactyly type A1 (OMIM# 174200; Radhakrishna et al., 1997). This condition is the result of an autosomal trait that is characterized by an extra digit in the ulnar and/or fibular side of the upper

and/or lower extremities. The extra digit is usually well formed, and it articulates with the fifth or extrametacarpal/metatarsal bone. Thus, different truncated GLI3 proteins are associated with different clinical syndromes, thereby highlighting the different functions of specific GLI3 variant proteins during limb development. Because GLI2 is also required to transduce Hedgehog signaling, mutations in the human *GLI2* gene have also been found to cause postaxial polydactyly apart from developmental defects affecting the pituitary gland and the brain (Roessler et al., 2003).

DV limb patterning is controlled by Wnt7a/Lmx1b signaling in mice. In humans, there is evidence that the Fuhrmann syndrome (OMIM# 228930) and the Al-Awadi/Raas–Rothschild/Schinzel phocomelia syndrome (OMIM # 276820) are caused by mutations in the *WNT7A* gene (Woods et al., 2006). These two syndromes have been considered to have distinct limb malformations characterized by various degrees of limb aplasia/hypoplasia and joint dysplasia. It has been suggested that mutations causing a partial loss of WNT7A function lead to Fuhrmann syndrome, whereas null mutations lead to the more severe limb truncation phenotypes observed in Al-Awadi/Raas–Rothschild/Schinzel phocomelia syndrome. Again, the findings in human limb malformations provide insight into the role of WNT7A in multiple aspects of vertebrate limb development. Because LMX1B is required for determining dorsal limb identity in mice, mutations resulting in the haploinsufficiency of the human LMX1B gene have been found to cause the nail–patella syndrome (OMIM# 161200), which affects DV limb development and which is characterized by dysplasia of the nails and absent or hypoplastic patellae. Other limb features of the nail–patella syndrome include elbow stiffness with limitations involving pronation and supination. Consistent with the findings in the $Lmx1b^{-/-}$ mutant mice (Chen et al., 1998; Pressman et al., 2000) is the fact that mutations in the *LMX1B* gene also cause renal abnormalities and open-angle glaucoma (OMIM# 137760).

IV. CONCLUSIONS AND PERSPECTIVES

Studies of classic embryology and molecular genetics in model organisms have led to the identification of molecular pathways controlling coordinated three-dimensional limb development. These studies have also provided insights into the understanding of the molecular and pathologic mechanisms of congenital human limb defects. The general principles will be relevant to devising new approaches for tissue repair. In addition, because of the pleiotrophic effects of the limb development pathways in other developmental processes as what has been shown in mouse and human genetic studies, the developing limb has provided an excellent model system for understanding how different signaling pathways operate and interact with each other in embryonic development. Although many genes and pathways with roles in limb development have been identified, we are still far way from getting a full picture of how limb development is controlled at the molecular level. There are considerable gaps in our current understanding. To gain clarity with regard to the cellular basis of limb development, we also need to find creative new ways to visualize the behavior of extracellular signaling molecules and to measure their concentrations and the cellular responses they trigger. Furthermore, it is not yet clear how the three-dimensional positional information, which is controlled by

distinct signaling pathways, is integrated into the cells of the developing limb. Both the mouse and human genomes have been sequenced, and recent developments in functional genomic studies in mice (e.g., chemical and insertional mutagenesis and large-scale gene targeting) will provide a tremendous amount of new information for the understanding of limb development. These studies—in combination with the ever-improving and powerful techniques of the genetic mapping of human disease—have allowed for the advancement of our understanding of limb development at an unprecedented speed.

SUMMARY

Vertebrate limb development has been a fertile field for the understanding of the functional mechanisms of cell–cell signaling in the control of embryonic development. The quick advancement of the molecular genetic studies of vertebrate limb development has benefited tremendously from classic embryological experiments in the chick. These studies have revealed the molecular networks that control limb development in the following areas:

- Limb bud formation at specific position along the AP body axis may be determined by the expression of *Hox* genes.
- Limb initiation is controlled by the transcription factors Tbx4 (hindlimb) and Tbx5 (forelimb) and by the Wnt/Fgf signaling pathways.
- Limb outgrowth and patterning along the three axes (PD, AP, and DV) are controlled by three signaling centers (AER, ZPA, and non-AER limb ectoderm, respectively). Coordination among the three signaling centers is made through interactions among the signaling pathways that mediate the function of the respective signaling centers.
- Signaling pathways controlling limb development also often play critical roles in the regulation of other important developmental processes.
- Genetic variations in humans that disrupt limb development lead to congenital limb malformations and other defects.

GLOSSARY

Bardet–Biedl syndrome
A condition that is characterized mainly by obesity, pigmentary retinopathy, polydactyly, mental retardation, hypogonadism, and renal failure, in fatal cases.

Brachydactyly
A term which literally means "shortness of the fingers and toes (digits)." The shortness is relative to the length of other long bones and other parts of the body.

Gorlin syndrome
An autosomal-dominant cancer syndrome. Patients with this rare syndrome often have anomalies of multiple organs, many of which are subtle. Patients with Gorlin syndrome have the propensity to develop multiple neoplasms, including basal cell carcinomas and medulloblastoma, and they often demonstrate extreme sensitivity to ionizing radiation, including sunlight.

Extra digits on the hands or feet also occur among patients with this syndrome. Mutations in the *Patched* gene have been found to cause this syndrome.

Polydactyly
The anatomic variant that involves more than the usual number of digits on the hands and/or feet; also known as *hyperdactyly.* When each hand or foot has six digits, it is sometimes called *hexadactyly.*

Syndactyly
A condition in which the fingers fail to separate into individual appendages. This separation occurs during embryological development.

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39 SKELETAL DEVELOPMENT

PETER G. ALEXANDER,[1] AMANDA T. BOYCE,[2] and ROCKY S. TUAN[1]

[1]*Cartilage Biology and Orthopaedics Branch, National Institute of Arthritis, and Musculoskeletal and Skin Diseases, National Institutes of Health, United States Department of Health and Human Services, Bethesda, MD*

[2]*Musculoskeletal Diseases Branch, National Institute of Arthritis, and Musculoskeletal and Skin Diseases, National Institutes of Health, United States Department of Health and Human Services, Bethesda, MD*

INTRODUCTION

Because the skeleton is present only in vertebrates, the use of alternative model systems to study skeletal development is limited. Although useful paradigms and conserved cellular and molecular pathways in morphogenesis and development have been gained from studies of invertebrates, this chapter will focus on the genetics of skeletal formation in humans, mice, and chickens. The nonlethal nature of mutations that affect the skeleton results in a multitude of human syndromes as well as mouse knockout models that include skeletal dysmorphogenesis, which is vital to our understanding of skeletogenesis. The development of the Cre-lox conditional knockout system in mice has overcome lethal null and dominant-negative mutations in genes that affect multiple organ systems to reveal their function in skeletogenesis (Gu, 1993). The mouse genome can be manipulated to overexpress the proteins that are involved in skeletogenesis in a general or a tissue-specific manner as well. Experimentally, the chicken model is of great use, because it is easily manipulated *in ovo*, particularly during very early stages of limb bud outgrowth. Finally, zebrafish is a convenient model, because morpholino antisense technology conveniently allows scientists to alter gene expression (Nasevicius, 2000).

I. THE APPENDICULAR SKELETON

A. Origins of the Appendicular Skeleton

The formation of the lateral plate mesoderm and the initial outgrowth of the limb bud have been covered in Chapter 38, and a brief summary is provided

here (Capdevila, 2001). At a specified location along the axis of the body, cells from the lateral plate mesoderm migrate to the ectodermal surface of the embryo and begin proliferating, thereby forming the limb bud. The zone of polarizing activity and the apical ectodermal ridge (AER) determine the anterior–posterior and proximal–distal polarities of the developing skeletal elements, and they also influence cell proliferation and migration. As the limb bud grows larger, the mesenchymal cells most distant from the AER stop proliferating and condense, thus beginning the process of endochondral ossification that ultimately results in an adult bone. Endochondral ossification can be summed up as follows: mesenchymal cells condense, undergo chondrogenesis, hypertrophy, calcify, and apoptose. Blood vessels invade the space that is left behind by chondrocyte apoptosis, and osteoblasts and osteoclasts take up residence. Additionally, joints are created as single cartilage anlage segments, and they form cartilage where the two new skeletal elements articulate.

B. Chondrogenesis

1. Condensation of Limb Bud Mesenchyme

As the size of the limb bud increases, the AER will eventually be positioned such that the proximal mesenchymal cells will no longer be under the influence of the fibroblast growth factor (FGF) secreted by the AER. This change in FGF signaling, together with the Wnt and bone morphogenetic protein (BMP) gradients established during limb patterning, will signal the mesenchymal cells of the limb bud to begin the process of condensation. Mesenchymal cells are surrounded by an extracellular matrix (ECM) that is rich in hyaluronan, collagen type I, and an alternatively spliced form of collagen type II (IIA; Dessau, 1980; Maleski, 1996). Before condensation, cells begin to express hyaluronidase, an enzyme that digests the hyaluronan in the ECM, thereby denuding the mesenchymal cells and allowing them to communicate with each other via their cell surface proteins. At the same time, BMP-2, which is a member of the transforming growth factor-beta (TGFβ) superfamily that is present throughout the mesenchyme, turns on the expression of neural cadherin (N-cadherin; Oberlander and Tuan, 1994). N-cadherin on one cell binds with N-cadherin on another cell, thereby initiating a signaling cascade that is one of several that initiates condensation (Delise et al., 2002a; 2002b).

A common *in vitro* system used to study the condensation event is the high-density micromass culture of limb bud mesenchyme. Limb buds from day 4 chick embryos or day 11.5 mouse embryos are removed, digested, and the cells plated as high-density cell cultures. Over the course of a few days, these undifferentiated mesenchymal cells will condense and start differentiating into cartilage (Ahrens et al., 1977). The condensation event can be monitored with peanut agglutinin, which is a lectin that recognizes a galactosyl glycoprotein moiety on the surface of prechondrogenic cells (Aulthouse et al., 1987; Stringa and Tuan, 1996). Micromass cultures can be perturbed with neutralizing antibodies or infected with viruses carrying wild-type or mutated versions of proteins involved in early limb bud processes, thus elucidating the roles of these proteins. For example, treatment with anti–N-cadherin antibodies (Oberlander and Tuan, 1994) or infection with mutated N-cadherin prevents condensation, which ultimately inhibits chondrogenesis, thus demonstrating the importance of the condensation event (Delise et al., 2002a; 2002b). The importance of BMPs during early condensation has also been studied in

micromass cultures, but the more important studies have involved the retroviral infection of chick embryos *in ovo* or in mouse transgenic models. For example, the injection of retrovirus expressing the BMP inhibitor Noggin prevents condensation, thus demonstrating the necessity of BMP signaling during early skeletogenesis (Pizette et al., 2000). Similarly, mice overexpressing Noggin in their limbs fail to produce most of their skeletal elements (Tsumaki et al., 2002).

Other cell–cell interaction proteins, such as neural cell adhesion molecule (N-CAM), and multiple ECM proteins and their cell surface receptors are implicated in the condensation process. TGF-β initiates the expression of fibronectin (FN), which is an extracellular protein that is vital to condensation. There are multiple splice variants of FN, but two are particularly interesting with regard to chondrogenesis (White, 2003). Before condensation, FN expresses exon IIIA. Cells spread less in the presence of the IIIA exon, which allows the cells to round up; this process allows better packing, which positively influences condensation. After condensation is complete, exon IIIA is spliced out. Versican is another ECM protein expressed in mesenchyme that, like FN IIIA, prevents cell spreading (Williams et al., 2005). In addition to fibronectin and versican, the thrombospondins (including cartilage oligomeric matrix protein [Kipnes et al., 2003]), the tenascins, and many other ECM proteins are necessary for proper condensation.

At this time of development, another member of the TGF-β superfamily, growth/differentiation factor 5 (GDF-5), is expressed (Chang et al., 1994, Storm et al., 1994). In micromass models, GDF-5 regulates condensation by increasing the ability of cells to communicate via gap junctions. Connexin 43 is expressed in developing limbs in an overlapping pattern with GDF-5, which suggests that connexin 43 may be the dominant gap-junction protein (Coleman et al., 2003a; 2003b). The overexpression of GDF-5 by infecting the chick limb *in ovo* with GDF-5 constructs leads to larger cartilage elements with increased cell numbers. It is proposed that the increased number of cells is the result of increased cell adhesiveness as opposed to an increase in proliferation (Francis-West et al., 1999). In mice that are null for GDF-5, chondrocyte condensation size is significantly reduced. Because the size of the initial condensations regulates the size of the future skeletal elements, these mice are severely dwarfed. Human mutations in the GDF-5 gene lead to a spectrum of dwarfisms, the most severe of which leads to a near absence of fingers.

The Sry-related HMG box-containing transcription factor Sox9 is critical for every step of chondrogenesis, and it is widely used as the marker to detect chondrocytes. Using the Cre-lox system, Sox9 can be eliminated specifically in early mouse limbs before condensation (Akiyama et al., 2002). In these animals, no condensations form in the limb bud, and ultimately these animals are born with a complete absence of limbs. In micromass cultures created using $Sox9^{-/-}$ chimeric limb buds, $SOX9^{-/-}$ cells cannot be found in condensing regions. In $SOX9^{-/-}$–wild-type chimeric animals, $SOX9^{-/-}$ cells are excluded from the cartilage primordium (Bi et al., 1999). The introduction of a bead soaked in BMP-2 into the developing chick limb leads to an upregulation of Sox9 expression, which suggests that the BMPs are important for the initiation of chondrogenesis (Healy et al., 1999). Although the exact mechanism of the Sox9 induction of condensation is unclear, its role in the initiation of overt chondrogenesis is well understood, and it will be covered in detail in the next section of this chapter.

The size of the condensation is critical during skeletogenesis. A condensation that is too small may fail to undergo chondrogenesis; if it is too large, the final bone will also be too large. The early cartilaginous skeleton determines the size and shape of the future bones, and thus its development must be very well controlled. Growth factors and the proteins responsible for cell–cell and cell–matrix interactions are critical for determining the size of the condensations by altering cell adhesion and migration. However, the cells in the condensations also proliferate, and the proteins that guide proliferation are thus noteworthy. Homeobox genes have been implicated in the control of the proliferation of the early mesenchyme (Boulet et al., 2003, Goff et al., 1997). HoxA11/HoxD11 double mutant mice have shortened forelimbs as a result of decreased FGF expression in the AER (Boulet et al., 2003). Because FGFs in the AER direct outward growth, any changes in FGF expression will alter limb bud growth. Interestingly, Hox genes are activated by BMPs, which further demonstrates the importance of FGFs in skeletogenesis (Duprez et al., 1996).

At the stage of mesenchymal condensation, the growing limb bud contains mesenchymal cells that have begun to adhere to one another and to communicate, thereby readying themselves for differentiation into chondrocytes. Before that happens, the edges of the condensation must be defined (Hall et al., 2000). Multiple cell–matrix interactions are thought to be involved in this process, but we will focus on just one interaction: the one that occurs between syndecan and FN (Figure 39.1). Syndecan is an integral membrane proteoglycan that is found in the mesenchymal cells that surround the initial condensation. Syndecan binds to FN, which is found in the ECM that surrounds cells during the early condensation. The interaction of syndecan and FN leads to an intracellular signaling cascade that instructs the mesenchymal cell to downregulate N-CAM. The point at which cells expressing N-CAM meet cells that are not expressing it becomes the boundary between the future cartilage anlage and the surrounding mesenchyme. Along this boundary, mesenchymal cells will flatten and become the perichondrium, which is the thin layer of cells that surrounds the cartilage skeletal elements that will later become the periosteum.

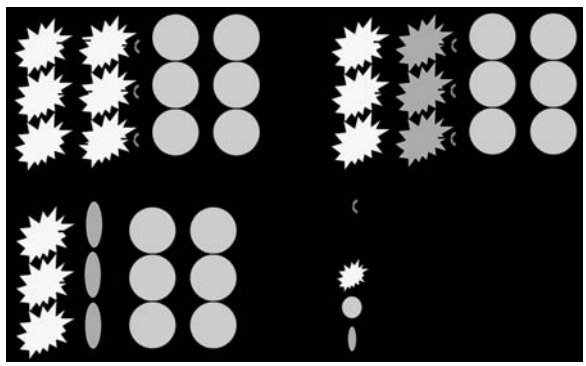

FIGURE 39.1 Establishment of the mesenchymal condensation boundary in the developing limb bud. A, Syndecan on the mesenchymal cells binds to fibronectin in the extracellular matrix of cells during early condensation. **B,** The interaction of syndecan and fibronectin leads to a downregulation of cell adhesion molecules such as neural cell adhesion molecules. **C,** Cells that no longer express neural cell adhesion molecules flatten and become the perichondrium, which is the thin layer of cells that surrounds cartilage.

2. Cartilage Differentiation

As stated previously, the primary gene responsible for driving the conversion of mesenchymal cells to chondrocytes is Sox9, and it does this by turning on two other Sox genes: Sox5 and Sox6 (Akiyama et al., 2002). In animals lacking Sox9, Sox5 and Sox6 are never expressed; Sox5 and Sox6 are known to have Sox9 binding sites in their enhancers, thereby explaining this finding. Sox5 and Sox6 are redundant, and thus mutations in either gene lead to no overt phenotype. However, animals lacking both Sox5 and Sox6 have severely reduced long bones (Smits et al., 2001). Sox9 also binds the enhancer regions of collagen types II and XI, and it turns on their expression early during chondrogenesis (Bi et al., 1999). Later, together with Sox5 and Sox6, Sox9 increases the expression of collagen type IX, aggrecan, and link protein (Lefebvre et al., 1998). The expression of these ECM proteins is an indicator of mature cartilage. Collagen type II is the dominant fibril form of collagen in cartilage, and it is crosslinked by collagen type IX. Mutations in either collagen type II or IX lead to disorganized growth plates and early onset osteoarthritis (Cremer et al., 1998). Aggrecan is a sulfated proteoglycan that is important in the maintenance of the high water content of cartilage (Dudhia et al., 2005). These structural components are absolutely vital to the maintenance of the mechanical properties of cartilage. This is particularly important in the articular cartilage at the ends of bones in joints. In addition, growth factors can be sequestered in the ECM, and cell surface receptors have important interactions with ECM proteins.

3. Growth Plate Regulation and Long Bone Development

The growth plate is an orderly arrangement of chondrocytes as they proliferate, differentiate, and apoptose, and they leave behind a mineralized matrix for osteoblasts to invade (Figure 39.2, A). A well-functioning growth plate is necessary for the lengthening of the long bones, and any perturbations

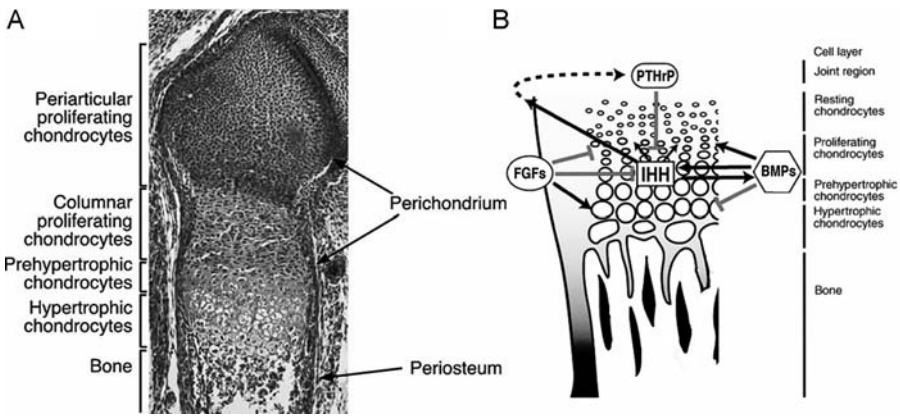

FIGURE 39.2 **Regulation of cartilage growth plate maturation. A,** The growth plate is an orderly arrangement of chondrocytes as they proliferate, differentiate, and apoptose, leaving behind a mineralized matrix for osteoblasts to invade. **B,** The Indian hedgehog/parathyroid-hormone related protein negative feedback loop and bone morphogenetic protein/fibroblast growth factor antagonism: Indian hedgehog and bone morphogenetic proteins increase proliferation and prevent hypertrophy, whereas fibroblast growth factors prevent proliferation and promote hypertrophy. (Adapted from Goldring et al., 2005, and Kornak et al., 2003. See color insert.)

of the system will lead to gross deformities. The growth plate is separated into resting, proliferating, prehypertrophic, and hypertrophic cells. Each bone initially has two growth plates, with their resting chondrocytes toward the epiphyses and their hypertrophic zones meeting in the diaphysis. As the bone becomes larger, secondary growth plates called *secondary ossification centers* form in the ends of the long bones. Growth plates are present before birth, and they stay active until the end of puberty, when growth is complete.

Sox5 and Sox6 are thought to initiate the formation of the growth plate by inducing proliferation between the epiphysis and diaphysis in the areas in which the columns of proliferating cells will appear (Smits et al., 2004). In animals that are null for Sox5 and Sox6, there are no columnar cells present, and growth plates fail to form. After the proliferating columnar cells are established, the cells at the medial edge must mature into prehypertrophy. The rest of this section will illustrate the carefully balanced act of proliferation versus hypertrophy. Sox5 and Sox6 prevent prehypertrophy at least in part by inhibiting the expression of FGF receptor 3 (FGFR-3). The FGFs prevent proliferation, and, in many tissues, cells either proliferate or differentiate. Thus, to prevent proliferation is to promote differentiation. How the medial edge differentiating cells escape the Sox5 and Sox6 proliferation cues is unclear, but it is likely that there are soluble signals that mediate this conversion.

Upon the initiation of hypertrophy, the cells are committed and will ultimately undergo apoptosis. After the first prehypertrophic cells are established, they begin secreting signals that will interact with proliferating cells, thus setting up the cross talk that balances proliferation and hypertrophy. The Indian hedgehog (Ihh)/parathyroid hormone related protein (PTHrP) negative feedback loop and BMP/FGF antagonism will be covered in more detail to illustrate this point (see Figure 39.2, B). Ihh is a secreted morphogen that is important in both limb bud patterning and growth plate regulation. Ihh null animals show decreased proliferation of the growth plate chondrocytes as well as an increase in hypertrophy. The misexpression of Ihh leads to a decrease in hypertrophy (St-Jacques et al., 1999). PTHrP is a secreted protein that shares homology and a receptor with parathyroid hormone. Like the Ihh knockout, the PTHrP null animal exhibits increased hypertrophy (Amizuka et al., 1994). The misexpression of PTHrP gives rise to a completely cartilaginous skeleton, which indicates a complete absence of hypertrophy. The expression patterns of the proteins involved in the Ihh and PTHrP signaling pathways have been established. Postmitotic, prehypertrophic cells express Ihh. The Ihh receptor Patched is found in the perichondrium surrounding the growth plate as well as in the proliferating zone. The Ihh activation of Patched in the perichondrium turns on the expression of TGF-β, which in turn activates PTHrP expression, also in the perichondrium (Alvarez et al., 2001; 2002). The PTHrP receptor is found only in proliferating cells on the edge of prehypertrophy. PTHrP acts on the proliferating cells to prevent them from entering into hypertrophy. In addition, Ihh can act directly on the cells of the proliferative zone to stimulate proliferation. Taken together, Ihh, in concert with PTHrP, acts in a negative feedback loop: cells committed to hypertrophy secrete a protein that prevents hypertrophy in proliferating cells.

The story becomes more complicated with the addition of BMP/FGF antagonism and its control over the Ihh/PTHrP feedback loop (Minina et al., 2001; 2002). FGF-2, -8, -9, -17, and -18 are all found in cartilage,

although their actions are dependent on which receptors they bind and where those receptors are located. FGFR-3 is found in proliferating and prehypertrophic cells, whereas FGFR-1 is found only in hypertrophic cells. Of the FGF knockout animals, only the FGF-18 null animal has an abnormal skeletal phenotype, and it closely resembles the FGFR-3 knockout. Both have expanded proliferative and hypertrophic zones, although the FGF-18 null animal also has delayed bone formation, which indicates that it may also signal through FGFR-1. FGFs have been shown to decrease proliferation by direct action as well as by decreasing Ihh expression. Decreased Ihh expression increases differentiation. FGFs not only increase commitment to hypertrophy, they also accelerate hypertrophy by directly acting on hypertrophic cells. Presumably the effects on Ihh expression and on proliferation are mediated by FGFR-3, although the latter effect is also mediated by FGFR-1.

The BMPs are present throughout the cartilage and the perichondrium. They are known to increase proliferation directly and by the upregulation of Ihh. In addition, they inhibit hypertrophic differentiation independently of the Ihh/PTHrP signaling cascade. Although they upregulate Ihh, Ihh in turn upregulates them. Quite obviously, the effects of FGF and BMP are directly opposite one another, and they operate by regulating the expression of Ihh as well as by independent means. In limb culture systems, adding FGF-2 and BMP-2 together induces no change to the bones, whereas, independently, FGF-2 increases hypertrophy, and BMP-2 increases proliferation. Interestingly, the addition of BMP-2 to limb cultures expressing constitutively active FGFR-3 rescues the phenotype (Minina et al., 2001; 2002). This is particularly important because FGFR-3–activating mutations are responsible for achondroplasia, which is the most common form of human dwarfism (L'Hote et al., 2005).

Another protein that is critical to the differentiation of chondrocytes to hypertrophy is core-binding factor 1, which is also known as runt-related transcription factor 2 (RUNX; Otto et al., 1997; Fugita et al., 2004). RUNX2 is found in prehypertrophic and hypertrophic chondrocytes as well as in osteoblasts. The primary phenotype of mice that are null for RUNX2 is a failure to form bone as a result of an absence of osteoblasts. However, infecting chick or mouse chondrocytes with antisense constructs for RUNX2 prevents chondrocyte hypertrophy, thus confirming its role in endochondral ossification. In addition, the infection of immature chondrocytes with RUNX2 accelerates the rate of hypertrophy. This acceleration of hypertrophy is the result in part of the upregulation of collagen type X and metal metalloproteinase 13 (MMP-13), which will be discussed later (Enomoto et al., 2000; Zheng et al., 2003). In addition, RUNX2 upregulates vascular endothelial growth factor (VEGF), which is necessary for the vascular invasion of the hypertrophic zone.

Growth hormone (GH) and insulin-like growth factors (IGFs) are important for controlling the length of long bones, although their targets, molecular effects, and expression patterns are as yet unclear (Robson et al., 2002). GH is responsible for gigantism and dwarfism, because of over- or underexpression, respectively. It is known that GH is released from the pituitary gland and that it stimulates the liver to release circulating IGF-1. Eliminating liver-derived circulating IGF-1 stunts growth, which suggests that the liver is the source of chondrocyte-activating IGF. Alternatively, directly injecting GH into the tibia increases length both by directly influencing resting chondrocytes to proliferate and by increasing the local expression of IGF-1. Interestingly, the

growth plates of IGF-1 null mice have no alteration in cell proliferation, but they show a decrease in hypertrophic cell height. This finding suggests that perhaps IGF-2 is important in the stimulation of proliferation. Complicating matters further is the fact that multiple IGF binding proteins, which inhibit the activities of the IGFs, as well as the two IGF receptors are found in overlapping patterns throughout the growth plate. Although it is clear that the GH/IGF system is important in long bone length, more work needs to be done to clarify the process.

The term *hypertrophy* has been mentioned multiple times, but it has not been defined. Hypertrophic cells are easily identified histologically, because they are the largest cells of the growth plate as a result of an increase in intracellular organelles. In fact, the increased height of hypertrophic cells is responsible for more than 50% of bone lengthening. In molecular terms, gene expression of collagen type X and alkaline phosphatase defines hypertrophy. Both of these proteins are important in mineralization. Collagen type X is thought to influence mineralization by stimulating calcium accumulation in matrix vesicles and by acting as a docking site for the matrix vesicles (Kwan et al., 1997). Alkaline phosphatase is found in matrix vesicles, and it leads to an accumulation of pyrophosphate, which is a mineral that is important in the formation of calcium hydroxyapatite crystals. Together, collagen type X and alkaline phosphatase act to initiate the mineralization of cartilage, which prevents any further growth in the hypertrophic zone of cartilage and sets up a scaffold for osteoblast invasion.

After cells undergo hypertrophy, their matrix is remodeled, they undergo apoptosis, and the cartilage model is invaded by capillaries, although not necessarily in that order. It is believed that matrix remodeling by MMPs is the initiator of apoptosis and vascular invasion. Mouse knockout models for both MMP-9 and MMP-13 show a reduction in angiogenesis, ECM remodeling, and apoptosis (Stickens et al., 2004; Vu et al., 1998). MMP-13, which is turned on by cbfa1, cleaves collagen type II, which is still highly expressed in the hypertrophic zone. After the degradation of collagen type II, VEGF previously expressed under the influence of RUNX2 and sequestered in the ECM is released. VEGF acts as an activator of vasculogenesis as well as a recruiter of osteoclasts (Zelzer et al., 2002). Osteoclasts will be covered in detail later, but for now it is important to know that their primary function is ECM degradation. Osteoclasts express MMP-9, which further processes the collagen type II initially cleaved by MMP-13 and which, together with MMP-13, acts to degrade aggrecan. There are two requirements for capillary invasion: proangiogenic growth factors and space. The actions of MMPs provide both. In addition, MMPs are responsible for promoting apoptosis. Either directly or indirectly, MMP action either releases a proapoptotic factor from the ECM or the ECM protein fragments stimulate the cells to apoptose.

C. Osteogenesis

1. Differentiation

After a hypertrophic zone is established in the growth plate, some cells in the perichondrium surrounding the newly hypertrophic cells begin to differentiate into osteoblasts (bone-forming cells), and the bone collar is formed. There are at least three genes that are known to be important in this conversion: Ihh, RUNX2, and osterix. In addition, the BMP signaling pathway is

critical to bone formation. Ihh is secreted by prehypertrophic cells, but the Ihh receptor Patched is expressed throughout the perichondrium. In Ihh null mice, there are no osteoblasts, which highlights how vital this protein is in bone production (St-Jacques et al., 1999). Ihh affects differentiation by working upstream of the transcription factor RUNX2 as well as by increasing expression of BMPs. Animals that are null for most BMPs are early embryonic lethal, although BMP-6 and BMP-7 knockouts have mild skeletal phenotypes (Jena et al., 1997; Solloway et al., 1998). The ectopic expression of the BMP inhibitor Noggin or dominant-negative BMP receptors leads to decreased osteoblast function and differentiation. The ectopic expression of BMP-2 and BMP-4 leads to heterotopic ossification. In fact, fibrodysplasia ossificans progressiva, which is a human disease caused by the ectopic activation of the BMP pathway via mutations in an activin receptor (ACVR-1), leads to horrible disfigurations as ossification occurs throughout the soft tissues of patients' bodies (Shore et al., 2006). BMPs are also capable of upregulating RUNX2, possibly acting as the effector of the Ihh upregulation of RUNX2.

RUNX2 is the master switch that differentiates perichondrial precursor cells into preosteoblasts (Otto et al., 1997; Fugita et al., 2004). Preosteoblasts do not form bone, but they do express osteoblast specific proteins. They also express chondrocyte-specific genes, which suggests that they are not yet irreversibly committed to the osteoblast lineage. RUNX2 acts upstream of osterix, which is another transcription factor that is involved in osteoblast differentiation. Although RUNX2 null mice do not express osterix, the effect is not thought to be direct. Alternatively, BMP-2 is known to directly activate osterix expression. Osterix is responsible for converting preosteoblasts into bone-forming osteoblasts (Nakashima et al., 2002). Mice lacking osterix have completely normal growth plates that vascularize appropriately. However, they fail to form bone.

Finally, communications between the perichondrial cells and the invading capillaries are required for the differentiation of osteoblasts. In one study, investigators placed cartilaginous long bones, complete with perichondrium, into the kidney capsules of host animals (Colnot et al., 2004). When a filter was placed around the anlage such that the vasculature of the kidney could not invade, maturation proceeded normally until osteoblasts were expected to differentiate and form bone. Although endothelial cells exist in the perichondrium, they can only form vessels and invade the hypertrophic zone upon contact with preexisting blood vessels. In other words, the cells of the perichondrium are not sufficient for osteogenesis. Communications between preexisting capillaries and the perichondrium are essential for osteoblast differentiation. Ultimately, VEGF is responsible for this finding, because it directs vasculogenesis.

2. Osteoclasts and Bone Remodeling

Osteoclasts were previously mentioned briefly, and they were described as being responsible for ECM digestion. They are important early in bone development, when the hypertrophic zone is remodeled for capillary and osteoblast invasion. For the rest of an organism's life, osteoclasts will work hand in hand with osteoblasts to digest and rebuild the skeleton. In fact, every year, 10% of the skeleton is digested and remodeled, thus resulting in a new skeleton every 10 years. This skeletal digestion must be carefully controlled, because too

much can lead to osteopenia, osteoporosis, or Paget's disease, and too little can lead to osteopetrosis. Bone remodeling is not just meant to repair and refresh the skeletal structure; it also regulates serum calcium and phosphate homeostasis, because bone is the body's storage bin for both elements. This additional level of complexity relies on communication with the gut, kidney, thyroid, and parathyroid (Boyle, 2003).

Osteoclasts derive from circulating monocytes that commit to the macrophage lineage. A small percentage of these circulating cells find their way to the bone and adhere. Interaction with bone stromal cells (preosteoblasts) is necessary for them to differentiate into osteoclasts. Two cell surface proteins produced by the osteoblasts, colony stimulating factor 1 (CSF-1) and RANK ligand (RANKL), and an unidentified serum factor are required for osteoclastogenesis. CSF-1 alone promotes proliferation, whereas CSF-1 and RANKL together lead to differentiation, survival, and fusion. Osteoclasts are multinucleated, and fusion is a prerequisite for function. Continued exposure to RANKL leads to osteoclast activation. Mice deficient in RANKL or RANK (Dougall et al., 1999), which is the cell surface protein on osteoclasts that binds RANKL, have a complete absence of osteoclasts, whereas other monocyte-derived cells are normal (Boyle et al., 2003).

Activated osteoclasts express a host of proteins that are involved in the degradation of bone and mineralized cartilage. To degrade bone, the osteoclast adheres to the bone surface, leaving a space into which it will secrete an acidic cocktail of digestive juices. To create this tight seal, the cell must express $\alpha v\beta 3$ integrin, which attaches to the collagen in bone. A vacuolar ATPase then pumps protons into the space between the osteoclast and bone, decreasing the pH to around 4.5 and allowing for the dissolution of the inorganic crystals in bone. Tartrate-resistant acid phosphatase and cathepsin K are also secreted into the space, and they are responsible for digesting the organic components of bone (Boyle et al., 2003).

Although RANK and RANKL are essential for osteoclast differentiation and activation, other signaling molecules influence osteoclastogenesis. Osteoprotegerin is a decoy receptor for RANKL, and it functionally blocks the interaction of RANK and RANKL. Osteoblasts can be stimulated to produce osteoprotegerin by estrogen and BMPs. Estrogen also downregulates the expression of RANKL in osteoblasts as well as the expression of the proinflammatory cytokines interleukin 1, interleukin 6, and tumor necrosis factor α, all of which positively influence osteoclast activity. Calcitonin, which is a protein that is secreted by the thyroid when the serum calcium level is elevated, prevents osteoclasts from attaching to bone. Alternatively, when the serum calcium level is low, the parathyroid gland releases parathyroid hormone, which stimulates osteoclast activity. Many additional factors, including vitamin D, corticosteroids, and PTHrP, positively regulate osteoclasts as well (Boyle et al., 2003)

Although fewer signaling pathways are known to negatively regulate osteoclast activity, one prominent example is estrogen. The impact of estrogen is particularly prominent in postmenopausal women, who frequently suffer from osteoporosis; this condition is characterized by a loss of bone mass, and it is caused by increased osteoclast activity without compensatory bone growth. Although osteoporosis is much more common in women, men are also susceptible. As men age, there is a reduction of testosterone. Testosterone is the precursor of estrogen, and it directly promotes osteoblast activity; thus,

both an osteoanabolic factor and an inhibitor of osteocatabolism are lost. Drug companies have developed pharmacologic agents that target osteoclast activity at multiple steps. Bisphosphonate, which stimulates osteoclast death, and estrogen treatments are most commonly prescribed, but drugs targeting osteoclast adhesion and digestion are also in use or in development (Boyle et al., 2003).

D. Joint Development

1. Interzone Development

The initiation of the synovial joint formation is still a mystery in developmental biology. The morphologic changes are well known, but the molecular controls are still being elucidated. It is known that, at specific points along the length of the cartilage anlagen, cells begin to flatten and form what is known as the *interzone* (Figure 39.3, A). These cells stop producing chondrocyte-specific proteins, particularly collagen type II and aggrecan, and they start producing collagen type I and hyaluronan. The cells at the center of the interzone begin to secrete an ECM that separates the cells, and this ultimately leads to cavitation. The remaining flanking interzone cells are thought to become articular chondrocytes. A mature joint consists of two opposing articular surfaces wrapped in a joint capsule and stabilized by ligament and tendon. Synovial cells line the inside of the joint, and they are responsible for producing joint fluid, which nourishes the avascular articular cartilage and lubricates the structure. Any perturbations in the joint structure, particularly as a function of age, lead to painful and often irreversible joint diseases, such as osteoarthritis and rheumatoid arthritis (Archer et al., 2003).

The location of the interzone is established quite early. If the presumptive elbow region is removed before any morphologic changes occur, then the joint fails to form. The specification of the location of the joint interzone is similar to the specification of the location of limb bud outgrowth in the early embryo. Not surprisingly, mutations in homeobox genes, which are known to be important during embryonic patterning, lead to fusions in the joints of the wrists, thus implicating them in this specification (Archer et al., 2003). Two novel homeobox proteins, Cux1 (Lizarraga et al., 2002) and Barx1 (Meech

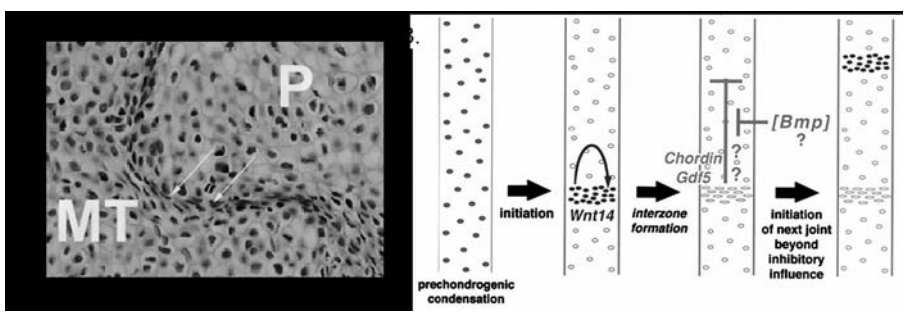

FIGURE 39.3 **Joint formation. A,** Morphologic description: cells of the interzone begin to flatten and stop producing chondrocyte-specific proteins (Archer, 2003). **B,** Molecular regulation: WNT-14 appears before the interzone is established, and it is required for interzone formation. Proteins expressed by the interzone, such as growth/differentiation factor 5 and chordin, prevent new joint initiation within a certain distance of an established joint. (Adapted from Archer et al., 2003, and Hartmann et al., 2001. See color insert.)

et al., 2005), have recently been found in early joints. Interestingly, the exogenous expression of Cux1 in the limb bud micromass system leads to the downregulation of chondrogenic markers, which is one of the first steps in interzone formation.

Concomitant with the establishment of the interzone, several growth factors and inhibitors appear. GDF-5, GDF-6, BMP-2, and chordin are all expressed in the interzone, whereas Noggin is specifically excluded. Upon cavitation, expression shifts to BMP-2, -4, -7, and chordin. The WNT proteins are candidate master genes that regulate gene expression in the interzone. WNT-14 is of particular interest (see Figure 39.3, B; Guo et al., 2004; Hartmann and Tabin, 2001). Although WNT-4, -5a, -14, and -16 are all found in joints, only WNT-14 is confirmed to have a direct effect on the presumptive joint. How WNT-14 signals is controversial, but its effects are agreed upon: it upregulates the expression of growth and differentiation factor 5 (GDF-5), chordin, and CD-44, and it downregulates Sox9 and Noggin. If exogenous WNT-14 is retrovirally expressed by virus, the region adjacent to the infection fails to form a joint where a joint would normally be. This implies that WNT-14–stimulated proteins act to prevent new joint initiation within a certain distance of an established joint. In other words, WNT signaling may be responsible for joint specification. However, at least one "initiator" joint would have to be established to begin this cascade of events.

The roles of the secondary signaling molecules are still vague, but some hypotheses for their functions have been suggested. The downregulation of Sox9 is responsible for the phenotypic switch of cells from chondrocytes to interzone cells. BMPs are thought to increase the expression of hyaluronan (HA), which is an extracellular glucosaminoglycan. HA binds to CD-44, which is a receptor on the surface of interzone cells. HA can act to condense cells or separate them, depending on the quantity of HA that is produced. Interzone cells produce large quantities of HA, and this leads to cell separation, which sets the joint up for cavitation. BMPs are also thought to cause apoptosis in the interzone, thus further demonstrating their role in cavitation. HA and CD-44 upregulation is also dependent on movement. The mechanical stimulation of cells increases CD-44 and HA expression, and paralyzed animals form interzones, but they fail to cavitate (Archer et al., 2003). Interestingly, GDF-5 decreases HA production. The overexpression of GDF-5 leads to joint fusion, which is thought to be the result of greatly decreased HA production and the failure to cavitate. How GDF-5 positively regulates joint formation is unclear, although it may be that it is important in a step between interzone formation and cavitation. GDF-6 is closely related to GDF-5, and, like GDF-5, it is expressed in a specific subset of joints; however, its effects are unknown. Chordin and Noggin are known to bind to and inhibit BMP2, -4, and -7 and possibly GDF-5. Curiously, chordin null mice have no joint phenotype, whereas Noggin-deficient mice fail to form joints (Brunet et al., 1998). This failure is thought to be the result of either a failure of GDF-5 to be produced in joints or of an increase in BMP bioavailability, which could drive the recruitment of cells into the cartilage model. That the interzone expresses agonists and their antagonists in the same spatiotemporal pattern is curious. Because cells rarely produce substances in a wasteful manner, there are likely additional controls on BMP/GDF or Noggin/chordin action. For example, perhaps an additional protein that cleaves the inhibitors exists. The diffusion rate of the proteins may also be quite different, such that

BMPs may be able to signal quite distantly while the inhibitors are confined locally (or vice versa). Finally, how the proteins bind to the ECM may differ, with some being sequestered while others are left free to signal.

II. AXIAL SKELETON

A. Origins of the Vertebrate Skeleton

The vertebrate skeleton is the product of cells from three distinct embryonic lineages. The craniofacial skeleton is derived from the cephalic paraxial mesoderm and invading cranial neural crest cells; the axial skeleton is derived from paraxial mesoderm in the vicinity of the notochord and neural tube; and the appendicular skeleton is the product of lateral plate mesodermal cells. The primordia of these tissues are specified early during ontogeny. In the chick embryo, three germ layers are formed 12 hours after fertilization with the onset of gastrulation. In the Hamburger Hamilton stage 5 head process embryo (20 hours; Figure 39.4, A; Hamburger and Hamilton, 1951), several different mesodermal compartments can already be identified: the axial chordamesoderm that will give rise to the notochord and the paraxial, intermediate, and lateral plate mesoderms. Within the paraxial mesoderm, two major regions are recognized: the segmental plate, which forms along the length of the primitive streak on either side of the notochord, and cephalic mesoderm on either side of the chordamesoderm or head process (see Figure 39.4, A). The paraxial mesoderm of the trunk and the cephalic mesoderm, in combination with invading cranial neural crest, give rise to many tissues, including bone, muscle, tendon, and dermis. Although the axial skeleton is completely derived from the cells of the paraxial mesoderm, cranial neural crest cells contribute heavily to the craniofacial skeleton. These cells are formed during the specification of the cephalic neural plate from ectoderm, and they begin deepithelializing from the dorsal lip of the cephalic neural folds as they fuse to form the neural tube (see Chapter 26).

B. Somitogenesis: Axial Skeletal Patterning

Somites are transient embryonic structures that are critical in the realization of the metameric pattern characteristic of the vertebral column and its associated tissues. They are segmental units that bud from the segmental plate as the node passes rostrocaudally down the primitive streak (see Figure 39.4, B). In the node's wake, the notochord forms from the chordamesoderm, and the neural plate differentiates from the ectoderm, thickening, folding, and fusing to form the neural tube. After these events, somites form from the rostral end of the paraxial mesoderm through a mesenchymal–epithelial transition in a species-specific, time-dependent fashion. In the chick, a new somite is formed every 90 minutes. Because the development of the embryo proceeds rostrocaudally, several stages of somitogenesis can be observed in one embryo (see Figure 39.4, C). The mesenchymal–epithelial transition involves an initially loosely associated ball of cells (the condensed somite) that reorganizes into a spherical epithelial structure (the epithelial somite) and that ultimately differentiates (see Figure 39.4, D). There is a small population of cells inside the presomitic mesoderm that retain their mesenchymal organization to form the cells of the somitic core, which is called the *somitocoele*.

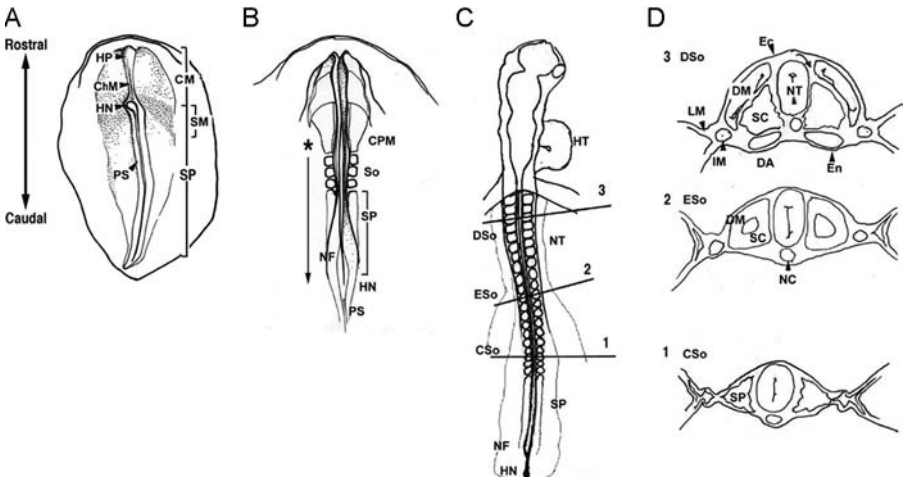

FIGURE 39.4 **A brief summary of somitogenesis in the chick embryo. A,** A head-process stage embryo after 20 hours of development. Two regions of paraxial mesoderm are identified: the segmental plate *(SP)* lateral to the primitive streak *(PS)* and the cephalic mesoderm *(CM)* lateral to the head process *(HP)*, which is a ridge that is formed by the underlying chordamesoderm *(ChM)* that also runs along the length of the embryo under the primitive streak. The chick node called Hensen's node (HN) is currently located at the rostral end of the primitive streak, and it defines the border of head and trunk structures. The thickening of the rostral segmental plate emanating posterolaterally from Hensen's node indicates the cellular condensation characteristic of somitic mesoderm *(SM)* that here will give rise to the first somite. **B,** A stage-6, 4-somite embryo in which morphogenesis of the neural folds *(NF)* has narrowed and elongated the embryo. The arrow indicates the rostrocaudal migration of Hensen's node *(HN)* down the primitive streak *(PS)* from its rostral extreme *(*)*. In the node's wake, neural folds have formed, and the segmental plate *(SP)* has lengthened, whereas somites *(So)* have formed from its rostral end at regular intervals. The cephalic mesoderm *(CPM)* has thickened with the invasion of neural crest cells, but it remains overtly unsegmented. **C,** A stage-13 embryo with defined brain vesicles, optic and otic cups, a folded heart tube *(HT)*, and approximately 20 somites. Hensen's node *(HN)* has almost reached the caudal end of the primitive streak *(PS)*. Because the development of the embryo proceeds rostrocaudally, somites at different levels of development can be visualized: condensed somites *(CSo, 1)*, epithelial somites *(ESo, 2)*, and differentiated somites *(DSo, 3)*. **D,** Transverse sections through the stage-13 embryo shown in **C** at different rostrocaudal levels as indicated in **C** and revealing **D1,** the condensed somite *(CSo)*; **D2,** the epithelial somite *(ESo)* with specified dorsolateral dermomyotome *(DM)* and ventromedial sclerotome *(SC)* induced by the notochord *(NC)*; and **D3,** the differentiated somite *(DSo)* in which the sclerotome has de-epithelialized, thus leaving the epithelial dermomyotome *(DM)* behind in the context of surrounding inductive tissues: notochord *(NC)*, neural tube *(NT)*, ectoderm *(Ec)*, endoderm *(En)*, intermediate mesoderm *(IM)*, lateral plate mesoderm *(LM)*, and the dorsal aortae *(DA)*. (Adapted from Alexander PG: *The role of paraxis in somitogenesis and carbon monoxide-induced axial skeletal teratogenesis*, PhD thesis, Thomas Jefferson University, Philadelphia, Penn, 2001.)

I. Somite Patterning

The process of somitogenesis is divided into several distinct phases: patterning, morphogenesis, differentiation, and the maturation of somite-derived tissues. Although the morphogenesis of the somite was described early in the history of embryology, the exact mechanism of the patterning process remains elusive. The regularity of somite architecture and formation during embryogenesis suggests that it must be controlled by a clock or a somitic oscillator within the embryo. The earliest experiments attempted to alter somite formation through surgical manipulations; these included removing individual tissues

from the embryo or observing individual tissues in isolation. Among the most important observations was that the segmental plate could undergo somitogenesis when separated from both the last previously formed somite and the axial structures (Packard, 1976). This finding demonstrated that segmentation does not require a continuous flow of information rostrocaudally and that the positional information or pattern for somitogenesis is established at gastrulation, possibly as the cells pass through the node to populate the paraxial mesoderm. Another enlightening observation was that implanted quail nodes could induce a secondary axis with a somite pattern dependent on the mediolateral position of the implant, thereby suggesting a possible morphogen emanating from Hensen's node (Horbruch et al., 1979). With the application of scanning electron microscopy, investigators identified segmentation of the entire paraxial mesoderm in a number and regularity that prepatterned the somite (Packard and Meier, 1983). These segments were called *somitomeres*.

Another important observation was that heat shock could induce somite abnormalities at regular intervals, thereby suggesting a timing-mechanism–based regulation of somitogenesis (Primmet et al., 1988). Similar segmentation defects were also observed when cell-cycle disruptors were applied to the embryo, which indicated a link between the cell cycle and the somite oscillator (Primmet et al., 1989). This led to the *clock and wavefront* model of somitogenesis, which invoked a molecular clock within the cells of the paraxial mesoderm that coordinated events in response to a signal wavefront moving rostrocaudally through it (Cooke and Zeeman, 1976). This quickly became the favored model of somitogenesis, and subsequent genetic studies have proven the central components of this theory to be true.

A major breakthrough in understanding the somitic oscillator was made with the cloning of the chick Hairy gene, c-Hairy1 (Palmeirim et al., 1997). Whole-mount *in situ* hybridization for cHairy1 mRNA revealed a dynamic caudorostral expression pattern in the segmental plate (Figure 39.5). During the 90-minute cycle of chick somitogenesis, c-Hairy1 expression begins within a broad domain in the caudal third of the segmental plate (see Figure 39.5, A), which slowly moves rostrally, becoming restricted to the posterior boundary of the next somite to be formed (see Figure 39.5, C). This was the first molecular evidence of a somitic oscillator within the segmental plate. These experiments established that the cycles of c-Hairy1 expression are autonomous to the paraxial mesoderm, occurring independently of surrounding tissues. These cycles are also independent of protein expression, which indicates that this particular gene responds to the putative somitic oscillator. Although its sequence suggests that it is a transcription factor, the function of c-Hairy1 remains elusive, as does its connection to the somitic oscillator.

2. Somite Border Formation

In the formation of the somite that follows, we recognize an initial condensed somite that is composed of unorganized mesodermal cells and its subsequent transformation into a spherical epithelial structure. These two processes are linked; however, different adhesion systems may be involved in each event. The boundaries of the somite are established through the Notch–Delta juxtacrine signaling system. Many components of this system exhibit cyclic expression that is autonomous to the segmental plate in phase with somite formation (Saga and Takeda, 2001; Kuan et al., 2004). The

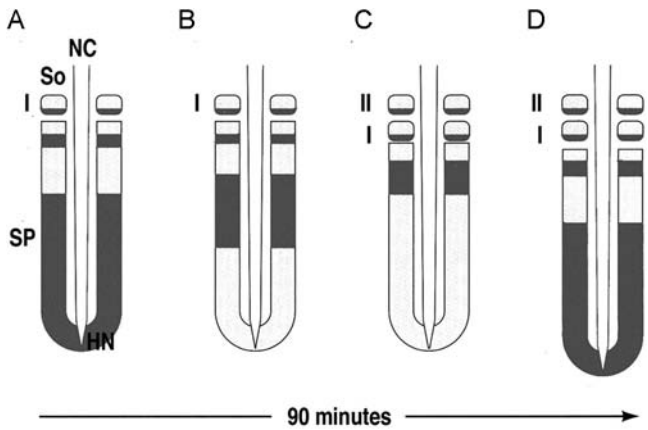

FIGURE 39.5 **Dynamic c-Hairy1 expression in the chick segmental plate. A,** The expression of c-Hairy in the caudal half of the segmental plate *(SP)*, in the posterior border of the last formed somite *(So)*, and in a region approximating the posterior half of the next somite at the beginning of the proposed somite oscillator cycle (time 0). Chick somites bud from the rostral segmental plate every 90 minutes; this is the temporal definition of the somite oscillator. **B,** Within 30 minutes, the caudal expression zone of c-Hairy1 expression is moving rostrally within the segmental plate, whereas the anterior zone is consolidating into a band prefiguring the posterior border of the next somite (somitomere). **C,** At 60 minutes, the posterior border of the next somite has formed, whereas the caudal c-Hairy1 expression zone has moved further rostral. **D,** The somite oscillator cycle is complete when c-Hairy1 expression returns to the caudal half of the segmental plate and an anterior zone prefiguring the posterior half of the next somite (as in **A**). *NC,* Notocord; *So,* somite; *HN,* Hensen's node. (Somite numbering system adopted from Christ et al., 1998, in which the last formed somite is always numbered with Roman numeral I. Adapted from Palmieram et al., 1997.)

importance of somite border formation in axial skeletal development is reflected in mutations of the Notch–Delta signaling system, which cause somite dysmorphogenesis and dramatic forms of scoliosis. For example, the *pudgy (pu)* mouse is characterized by a highly disorganized axial skeleton with many hemi and fused vertebrae and fused rib elements (Kalter, 1980; Kusumi et al., 1998). These abnormalities are prefigured by highly irregular somites in 9.5 to 10.0 post coitum *pu* embryos. The mutation was shown to be in DLL3, a heterotypic binding partner of Notch (Dunwoodie et al., 2002). Mutations in the human homolog of DLL3 are known to cause spondylocostal dysplasia, a severe congenital scoliosis with a disorganized vertebral column characterized by multiple hemivertebrae, fused vertebrae, and fused ribs.

Disturbances in the formation and maintenance of the axial skeleton lead to abnormal spinal curvatures in the neonate or adult. Abnormal axial skeletal curvature may be in the mediolateral or coronal plane (i.e., scoliosis) or in the dorsolateral or sagittal plane (i.e., kyphosis). However, most abnormal spinal curvature is mixed, with one direction of curvature being more dominant than the other. Scoliosis is the more common of these two abnormal curvatures, and it will be used as a model in this discussion. There are two major classes of scoliosis. Idiopathic scoliosis is a lateral curvature of the spine with no known cause. Depending on the definition of curvature, the incidence of idiopathic scoliosis may be as high as 2 to 3 cases per 1000 live births. It becomes evident postnatally, frequently during adolescence, and mostly in

females. Although the molecular genetics of idiopathic scoliosis remains unknown, there is a strong inheritable component. Many cases occur in the context of other musculoskeletal syndromes, including osteogenesis imperfecta, Marfan syndrome, Ehlers–Danlos syndrome, muscular dystrophies, cerebral palsy, and spinal trauma in which variations in the muscles and connective tissues that surround the axial skeleton cause imbalances in tension that lead to abnormal curvatures (Giampietro et al., 2003).

Congenital scoliosis is evident at birth, and it results from an underlying axial skeletal dysmorphogenesis. The incidence of congenital scoliosis is 0.5 to 1 case per 1000 live births, with a slightly greater incidence seen among males. Abnormalities of the vertebrae include hemivertebrae, wedge vertebrae, block vertebrae, vertebral bars, and butterfly vertebrae, among others. Hemivertebrae may be caused by ectopic or additional segments, whereas block vertebrae and vertebral bars are likely caused by a failure to produce or maintain segmentation (Erol et al., 2004). Although congenital scoliosis can be associated with genetic syndromes such as spondylocostal dysplasia, spondylocostal dysostosis, Alagille syndrome, Klippel–Feil syndrome, and Jarcho–Levin syndrome, up to 60% of congenital scoliosis cases result from an unknown cause (Giampietro et al., 2003). The involvement of other organ systems is common in these cases, which include spinal and neural tissues; urogenital, gastrointestinal, and cardiovascular tissues; and the craniofacial and appendicular skeletons. These syndromes include Klippel–Feil syndrome, Goldenhar's syndrome/OAV (oculoauriculovertebral dysplasia), VATER (vertebral anomalies, anal atresia, tracheo-esophageal fistula with esophageal atresia, and renal dysplasia), VACTERL (vertebral anomalies, anal atresia, cardiac malformations, tracheo-esophageal fistula, renal dysplasia and limb deformities) and the MURCS association (Mullerian duct aplasia-renal aplasia-cervicothoracic somite dysplasia; Table 39.1). In contrast with idiopathic scoliosis, these cases usually arise spontaneously, without a strong genetic component.

It has been hypothesized that the production and degradation of inhibitors or activators of Notch may reflect or constitute the somitogenic oscillator itself. Lunatic fringe is an excellent candidate, because its expression oscillates at the posterior border of the next somite to be formed (Figure 39.6; Evrard et al., 1998). Cells expressing Lunatic fringe can be transplanted into the caudal segmental plate and induce ectopic border formation. The transgenic overexpression of Lunatic fringe in the mouse embryo overwhelms endogenous Lunatic fringe oscillations in the segmental plate, and it inhibits all segmentation, thereby suggesting that the degradation of this component may constitute the somitic oscillator itself (Dale et al., 2003).

The concept of a wavefront in the clock and wavefront model implies an anterior–posterior gradient of a signal through the paraxial mesoderm. Two molecules with gradients of protein concentration in the paraxial mesoderm have been shown to be especially important in somitogenesis (Aulehla and Herrmann, 2004): FGF-8 (Sawada et al., 2001) and WNT-3a (Liu et al., 1999b). The message and protein of the first candidate wavefront molecule, FGF8, exhibits a caudorostral gradient in the segmental plate (see Figure 39.6; Dubrulle et al., 2001) that may be produced through an RNA decay mechanism (Dubrulle and Pourquie, 2004). Increasing the local concentration of FGF8 results in smaller somites, whereas the inhibition of FGF-8 signaling produces larger somites, thereby suggesting that this signal determines the

TABLE 39.1 Selected Human Syndromes with Scoliosis

Syndrome	Gene	Vertebral Abnormalities	Other Anomalies	OMIM #
Spondylocostal dysotosis and Jarcho–Levin syndrome	Dll3Mesp2Lnfg	Multiple hemivertebrae, fused vertebrae along the length of the spine, and fused ribs	Cranial neural tube defects, genitourinary defects	227300
Alagille syndrome	Jgd1	Butterfly vertebrae and shortened interpedicular space	Craniofacial, cardiac, and ocular defects; liver disease	118450
Klippel–Feil syndrome		Fused cervical vertebrae	Deafness, ear malformation, asymmetric facies	118110
MURCS		Shortened and fused vertebrae of the cervical and thoracic spines	Müllerian duct aplasia, unilateral renal aplasia	601076
VATER		Hemivertebrae and fused vertebrae, usually thoracic and lumbar and includes cases of lumbar–sacral agenesis	Anal atresia, tracheoesophageal fistula with esophageal atresia, renal dysplasia	192350
VACTERL		Hemivertebrae and fused vertebrae, usually thoracic and lumbar and includes cases of lumbar–sacral agenesis	Anal atresia, cardiac malformations, tracheoesophageal fistula, renal dysplasia, limb deformities	192350
Goldenhar syndrome/OAV		Fused vertebrae, usually cervical or thoracic	First and second branchial arch derivative hypoplasia, including facial clefts; esophageal atresia; cardiac, central nervous system, eye, and ear defects	164210

position of the somite boundary in response to a spatially progressive signal. The second candidate wavefront molecule, WNT-3a, is only expressed in the mesoderm as it migrates from the node (see Figure 39.6; Aulehla et al., 2003). A caudorostral gradient is possibly created through the extracellular degradation of this protein. In the spontaneous WNT-3a mutant mouse *vestigial tail (vt)*, FGF8 is downregulated in the tail buds and the segmental plate, thereby suggesting that FGF-8 acts downstream of WNT-3a (Greco et al., 1996). In either case, as the concentration of the gradient molecule is reduced

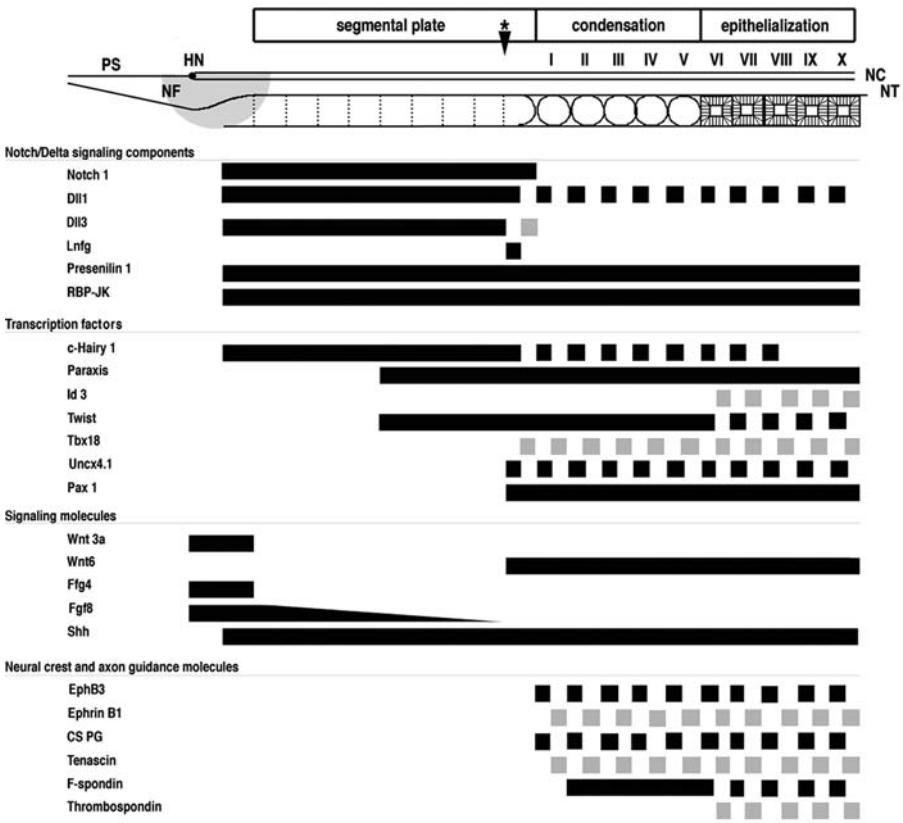

FIGURE 39.6 **Expression patterns of several important genes during somitogenesis.** A schematic representation of a chick embryo with various stages of somitogenesis, including the segmental plate with somitomeres, condensed somites *(I-V)*, epithelial somites *(VI-X)*, notochord *(NC)*, neural tube *(NT)*, neural folds *(NF)*, Hensen's node *(HN)*, and primitive streak *(PS)*. The asterisk indicates the posterior border of the next somite to be formed. The spatial gene expression pattern is indicated by shaded areas. (Adapted from Kuan et al., 2004.)

in more rostral positions, a threshold is reached in which border formation and epithelialization are permitted.

3. Somite Epithelialization

The epithelialization of the somite is dependent on a different signaling and adhesion system. Although the cyclic expression of somitogenic genes is observed in isolated segmental plates, overt somite morphogenesis is absent, because signals emanating from the overlying ectoderm are required (Figure 39.7). The removal of the ectoderm is known to inhibit somite formation, but it does not alter the cycling expression of the Notch–Delta system. The signal responsible for this activity is WNT-6, which is produced by the ectoderm overlying the segmental plate (Rodriguez-Niedenfuehr et al., 2003; Schmidt et al., 2004). An important transducer of WNT signaling is β-catenin, which acts both as a transcriptional regulator in the canonic WNT pathway and a component of focal adhesion complexes. During somitogenesis, β-catenin translocates to the plasma membrane, and it associates with N-cadherin as somitic cells increase their affinity for each other and form a spheric epithelium.

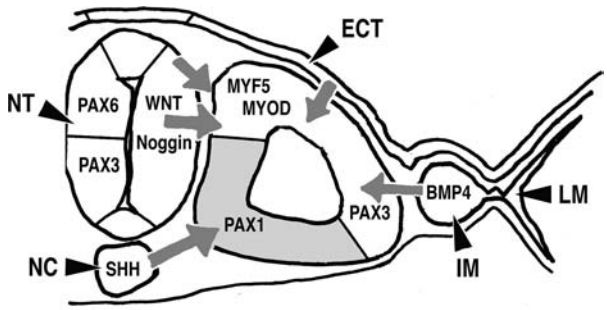

FIGURE 39.7 **Tissue interactions involved in the specification of the sclerotome in the ventromedial portion of the epithelial somite.** WNT-6 produced by the overlying ectoderm *(ECT)* induces the epithelialization of the somite. Sonic hedgehog emitted from the notochord *(NC)* and the floor plate of the neural tube *(NT)* induces Pax-1 in the sclerotome *(shaded region)*. Pax-1 expression is inhibited dorsally and ventrally by bone morphogenetic protein 4. MYF5, MYOD, and Pax-3 are maintained in the dorsolateral portion of the epithelial somite. *IM,* Intermediate mesoderm; *LM,* lateral plate mesoderm. (Adapted from Alexander PG: *The role of paraxis in somitogenesis and carbon monoxide-induced axial skeletal teratogenesis,* PhD thesis, Thomas Jefferson University, Philadelphia, Penn, 2001.)

The disruption of N-cadherin function inhibits somite epithelialization (Linask et al., 1998). This disruption may reflect the inhibition of β-catenin's transcriptional function and/or the inhibition of cadherin-based cell–cell adhesion via focal adhesion complexes. The interplay between the two functions of β-catenin is not clearly understood.

Somite epithelialization requires increased cell–cell and cell–matrix interactions in the segmental plate. Cadherins responsible for homotypic cell–cell adhesion play a vital role in this; this group includes several members, such as N-cadherin (Hatta et al., 1987), cadherin 11 (Kimura et al., 1995), and protocadherin (Kim et al., 2003). ECM molecules that are important in epithelial structures, such as FN and laminin, are also upregulated at this time (Solursh et al., 1979). These molecules are expressed in the rostral half of the segmental plate, and this coincides with the expression of several transcription factors, such as paraxis (see Figure 39.6); the expression of paraxis prefigures somite formation and epithelialization (Burgess et al., 1995; Barnes et al., 1997) and regulates β-catenin activity during somitogenesis (Linker et al., 2005). The knockdown and knockout of paraxis in various animal models have revealed both the importance of paraxis in epithelialization and the importance of the somite in organizing the axial tissues (Barnes et al., 1997; Burgess et al., 1996; Sosic et al., 1997). Although evidence of segmentation is still observed, somite differentiation in paraxis knockout mice is delayed and less precise, and this results in disorganized tissues. Homozygotes lacking paraxis die at birth as a result of an inability to breathe that is caused by the disorganization of the intercostal musculature (Burgess et al., 1996).

The expression pattern and function of axin2 suggest a link between the somitic gradients of WNT-3a and FGF-8 and the somitic oscillator that acts on the Notch–Delta signaling pathway (Aulehla and Herrmann, 2004). Axin2, which is a cytoplasmic component of canonic WNT pathways, is expressed in a caudorostral gradient under the control of WNT-3a. It is also expressed in a cyclic manner in the segmental plate at the posterior margin of the next somite to be formed, like Lunatic fringe. However, axin2 expression alternates with

Lunatic fringe (Aulehla et al., 2003). In mice that are null for the Notch ligand DLL1, Notch signaling is impaired, but axin2 oscillatory expression is still detectable. By contrast, the lack of axin2 in *vt* mice stops the oscillatory expression of Lunatic fringe but not its spatial expression pattern, which suggests a mechanistic link between the gradient and the clock. Axin2 downregulation during somite border formation may permit the influence of the canonic WNT-6 pathway on the segmental plate, thereby suggesting a mechanistic link with border formation and epithelialization as well. The results of microsurgical and gene knockdown techniques support this model; however, axin2 knockout mice show no axial skeletal defect. Alternatively, mutations in axin1 in the *fused (fu)* mouse produce profound axial skeletal dysmorphogenesis (Zeng et al., 1997). This discrepancy may reflect fundamental problems with previous experimental methods, the misinterpretation of phenomena, or a species-specific difference between mice and other developmental models.

D. Differentiation of the Sclerotome

After the patterning of the somite is accomplished, overt differentiation begins. The sclerotome that gives rise to the axial skeleton is derived from the ventromedial compartment of the somite (see Figure 39.4). During the differentiation of the sclerotome, the dorsolateral component of the epithelial somite, the dermomyotome, retains its epithelial structure, whereas the ventromedial sclerotome undergoes an epithelial–mesenchymal transition. This de-epithelialization is preceded by the expression of Pax-1 (see Figure 39.6; Barnes et al., 1996), a paired-box transcription factor that is disrupted in the *undulated* mouse (Balling et al., 1988), the phenotype of which includes scoliosis and missing vertebral bodies. The induction of Pax-1 expression is mediated by Sonic hedgehog (Shh), which is produced by the notochord and floor plate of the neural tube (see Figure 39.7; Johnson et al., 1994). Ablation of the notochord will prevent the expression of Pax-1 in the somite and the differentiation of the sclerotome. When a notochord or an Shh-soaked bead is implanted dorsal to the somite, Pax-1 is induced in the dorsal somite at the expense of myotomal markers (Brand-Saberi et al., 1993; Ebensberger et al., 1995). BMP-4 produced by the dorsal neural tube and intermediate mesoderm limits or modifies the effect of Shh to constrain sclerotome differentiation in the somite both dorsally and laterally (see Figure 39.4; McMahon et al., 1998; Tonegawa and Takahashi, 1998).

After the sclerotome delaminates, it migrates or relocates to the region around the notochord. The function of Pax-1 and other early markers of sclerotome, such as paired-box gene 9 (Pax-9) and mesenchyme forkhead 1 (MFH1), is to maintain the sclerotome by promoting proliferation and preventing cell death (Christ et al., 2004). Functionally, this maintains the sclerotome in a mesenchymal state, and it presumably prevents differentiation. As the sclerotome develops, the posterior half becomes increasingly dense, whereas the anterior half remains loosely populated; this reflects an important anterior–posterior polarity. The differentiation of the sclerotome begins in the posterior half in close association with the notochord. This is coincident with a downregulation of Pax-1 and -9 and an upregulation of Sox9, which is a master regulator of chondrogenic differentiation that controls the expression of major cartilage constituents such as collagen types II and X and aggrecan in a manner that is similar to that of long-bone development in the limb. The

wave of differentiation proceeds radially from the notochord dorsally to surround the neural tube and ventrally toward the ventral body wall. The sclerotomal cells that inhabit the area around the notochord will give rise to the centrum of the vertebral body. Other structures of the axial skeleton are also derived from the sclerotome, but they require additional regulatory mechanisms.

E. Neural Arch and Rib Development

An example of this alternative sclerotomal development is that of the vertebral neural arches that surround the spinal cord laterally and dorsally (Figure 39.8; Watanabe et al., 1998). The dorsal–medial sclerotome remains in a mesenchymal state, proliferating and migrating toward the dorsal ectoderm to surround the neural tube. These cells express MSX2, which maintains the mesenchymal phenotype of the sclerotome and which is required for subsequent chondrogenic differentiation (Monsoro-Burq et al., 1996; Monsoro-Burq and Le Dourain, 2000). This differentiation is dependent on BMP-4 signaling from the dorsal neural tube and the overlying ectoderm. The experimental dorsoventral rotation of the neural tube that removes the dorsal influence of BMP-4 on the dorsal sclerotome results in a failure of neural arch development (Nifuji et al., 1997).

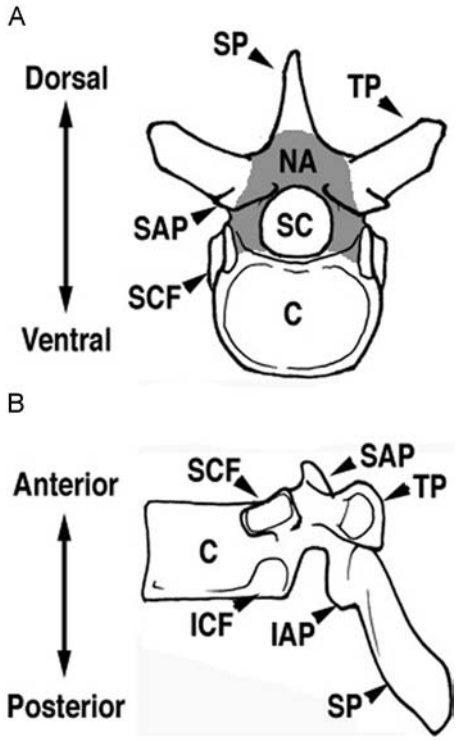

FIGURE 39.8 **Anatomy of the vertebral body. A,** Anterior view of a thoracic vertebral body. Important anatomic features include the centrum *(C)*, the neural arch *(NA)*, the transverse process *(TP)*, the spinous process *(SP)*, the spinal canal *(SC)*, the superior articular process or pedicle *(SAP)*, and the superior costal fovea *(SCF)*. **B,** Lateral view of a thoracic vertebral body. The gray area in **A** indicates a Pax-1–expressing sclerotomal origin. Additional anatomic features of a vertebral body include the inferior articular process or pedicle *(IAP)*, the costal fovea of the transverse process *(CFTP)*, and the inferior costal fovea *(ICF)*.

The transverse processes and ribs are also derived from the Pax-1–expressing sclerotome. The body wall in which the ribs reside is derived from the lateral plate that grows ventrally to encase the trunk and abdominal organs. During the time of rib mesenchyme outgrowth, Pax-3–expressing myoblasts and sclerotomal cells both migrate toward the expanding lateral plate mesoderm (Henderson et al., 1999). Quail–chick chimeras, in which the sclerotome or dermomyotome of a chick was replaced by homotypic quail compartments, showed that all parts of the transverse processes and ribs are derived from the sclerotome (Oliviera-Martinez et al., 2000; Evans, 2003). When the overlying ectoderm was removed from the body wall, rib development was severely impaired, which suggests an additional and uncharacterized inductive mechanism that regulates muscle and cartilage development (Sudo et al., 2001).

Much less is known about pectoral or pelvic girdle development, although errors in this cause disorders such as congenital hip dysplasia. Recent work looking at scapular development has revealed a dual origin: the blade of the scapula is derived from somitic mesoderm, whereas the head and neck are derived from the lateral plate mesoderm (Huang et al., 2000b; Ehehalt et al., 2004). The somitic cells that form the scapular blade come from the chondrogenic differentiation of dermomyotomal cells that are induced to express Pax-l. In contrast with the vertebral body, Pax-1 is induced (rather than inhibited) by BMP signals from the somatopleure (Wang et al., 2005). The identity of other factors important to the coordination of appendicular and axial skeletal articulation remains unknown.

F. Tendon Development

The bony elements of the axial skeleton develop in close association with the overlying musculature. The interaction is mutually dependent, and it is critical for the development of other connective tissues: the tendons and the ligaments. The characterization of a marker of ligament and tendon matrix, tenascin-C, has suggested that interactions between muscle and bone are important to the development of these connective tissues (Edom-Vovard and Duprez, 2004). Recent studies reported the discovery of a cellular marker for tendons and ligaments, scleraxis (a basic helix–loop–helix protein closely related to myogenic differentiation factor 1 [MYOD1] and paraxis), expressed throughout their development (Schweitzer et al., 2001; Brent et al., 2003). In the axial skeleton, scleraxis is first expressed at the cranial and caudal borders of the dorsalmost sclerotome in close proximity with the overlying myotome. This compartment has been named the *syndetome* (Brent and Tabin, 2002), and it is formed as a result of sclerotomal differentiation in a manner that is dependent on FGF-8 being emitted by the myotome. The removal of the myotome prevents scleraxis induction and tendon/ligament development, whereas an implanted FGF-8–soaked bead can rescue this deficit *in vivo* (Brent et al., 2004).

G. Resegmentation

A rostrocaudal polarity of the prevertebral sclerotome was first shown in 1855 (Remak, 1855 as cited in Stern and Keynes, 1987). Visually, one can see that the posterior half of the mesenchymal sclerotome is more dense than the anterior half. In addition, early embryologists identified a group of elongated and transversely oriented sclerotomal cells separating the rostral and caudal halves of the somite (von Ebner's fissures). The existence of these features confirms the

observation that the two halves of the epithelial somite have different adhesive qualities and that they have an influence on migrating neural crest cell and axons (Stern et al., 1986; Norris et al., 1989; Tosney, 1991). These cells migrate exclusively over the cranial portion (Oakley and Tosney, 1991). Somite anterior–posterior polarity is acquired from the very beginning with the differential localization of the Notch ligands DLL1 and DLL3, and it is reiterated by other molecules (see Figure 39.6). The polarity of the somites may serve to direct the migration of neural crest and neuronal axons and to organize other tissues associated with the somites in preparation for the resegmentation of the sclerotomes. Resegmentation is the process by which the bony elements are realigned out of phase with respect to other somite-derived tissues (i.e., muscles, tendons, ligaments, blood vessels) and the neural ganglia (Figure 39.9) to form a functional vertebral joint. Quail–chick chimeras have shown that, to form a new

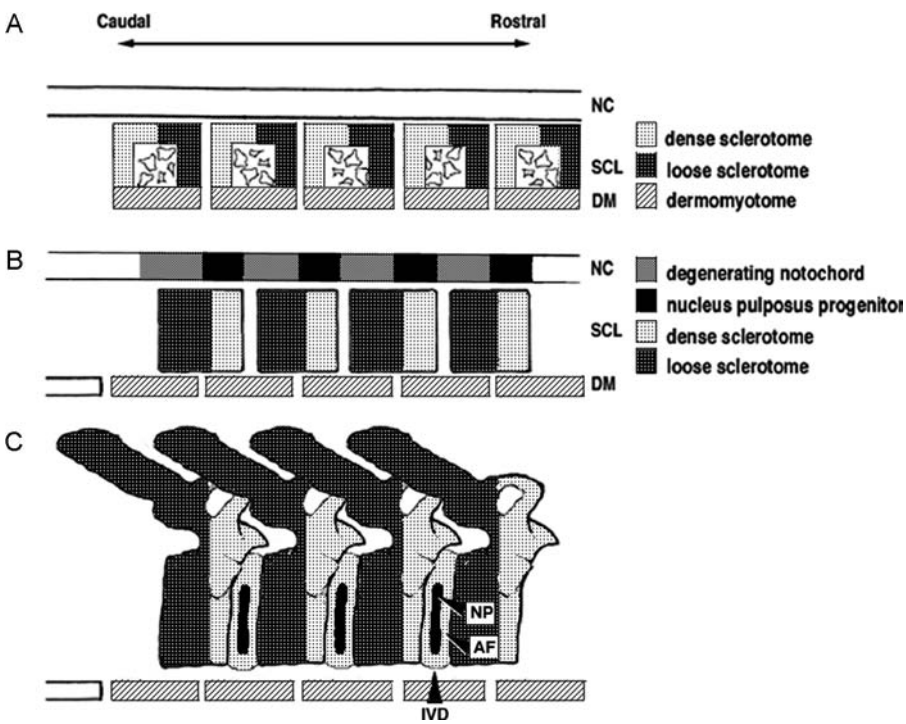

FIGURE 39.9 **Resegmentation of the somites in relation to the adult axial skeleton. A,** Schematic of epithelial somites with specified sclerotome *(SCL)* and dermomyotome *(DM, black)* and the caudal, more densely packed portion of the sclerotome *(medium grey)*. **B,** Schematic of resegmented, de-epithelialized sclerotomes with the densely packed sclerotome *(medium grey)*, which contributes to the annulus fibrosus of the intervertebral disc and possibly the anterior portions of the vertebral body. The loosely packed regions of the sclerotomes undergo chondrogenesis and endochondral ossification to form the majority of the vertebral body, including the centrum, the processes, and the ribs. Regional fates of the notochord *(NC)* are specified with light grey for the degenerating notochord neighboring the cartilaginous regions of the sclerotome and dark grey for the regions that will give rise to the nucleus pulposus of the intervertebral disc. **C,** Four thoracic vertebrae with the contribution of the posterior somites in **A** and corresponding densely packed anterior sclerotome to these vertebrae and annulus fibrosus *(AF; medium grey)*. The contribution of the notochord to the nucleus pulposus *(NP)* within the intervertebral disc *(IVD)* is highlighted in dark grey. The relationship of the vertebral bodies to their musculature derived from the dermomyotome is indicated in black.

segment, the posterior half of the mesenchymal sclerotome derived from one somite recombines with the anterior half of the mesenchymal sclerotome derived from the anterior somite (Huang et al., 2000a). In this way, the soft-tissue elements, including the muscles, now span the space between two vertebral bodies to produce an articulating joint (Bagnall et al., 1988; Bagnall, 1992; Aoyama and Asamoto, 2000). The loosely populated half of the new sclerotomal segment gives rise to the vertebral body (see Figure 39.9). The contribution of the more dense anterior half is not as clear. Part of this half does contribute to the bony vertebral body that protects the nerves of the dorsal and ventral ganglia and the blood vessels that supply a particular axial segment passing over and through it. The other portion contributes to the soft tissues and the annulus fibrosus of the intervertebral disc, although how this is accomplished remains unknown.

H. Intervertebral Joint

During the resegmentation process, the mesenchymal somitocoele cells once located within the epithelial somite are relocated to intervertebral space. Vital dye staining revealed that these cells or their progeny are involved in joint development. The removal of the original somitocoelic cells and their replacement with an acellular spacer resulted in fused vertebral centra and pedicles, thereby providing more evidence that these cells either give rise to the tissues of the vertebral joint or direct its development (Mittapalli et al., 2005).

The origin of the intervertebral disc, which is the specialized joint between the centra of two vertebrae (see Figure 39.9), is still controversial (Hunter et al., 2003). There are three components to the intervertebral disc: (1) the articular surfaces of the anterior and posterior vertebral bodies; (2) the annulus fibrosus, which is a specialized ligament that consists of concentric layers of collagen fibers that surround the third tissue; and (3) the nucleus pulposus, which is a more highly hydrated ECM-rich center of the intervertebral disc. As mentioned previously, the annulus fibrosis is derived from the densely packed sclerotomes after resegmentation. The origin of the nucleus pulposus, which absorbs the compressive forces along the axis, is elusive. In some vertebrates (e.g., chicks), the notochordal cells persist throughout life, whereas these cells disappear via apoptosis, terminal chondrocytic differentiation, or another phenotypic change in larger species (e.g., mice, humans). The original notochord-derived cells may be replaced by mesenchymal cells from the annulus fibrosis or other sources. Clinically, it has been conjectured that the loss of the notochordal cells is closely associated with the development of intervertebral disc degeneration and associated back pain.

I. Rostrocaudal Vertebral Identity

The axial skeleton is characterized by its metamerism, which is a pattern of similar—but not identical—repeating units. A comparison of a cervical, thoracic, lumbar, and sacral vertebra demonstrates the rostrocaudal variances. The regionalization of the somites and the constituent sclerotomes is determined by specific combinations of Hox genes in the paraxial mesoderm. In humans, there are 13 paralogous groups that are aligned in four clusters. These clusters are expressed in a colinear fashion rostrocaudally (3′ to 5′ along the genome), which suggests an epigenetic mechanism of gene regulation (Gruss and Kessel, 1991; Christ et al., 2000; see Chapter 9). Refinement of the

anterior–posterior expression of these genes may occur through the action of retinoic acid (RA), a physiologic metabolite of vitamin A that is expressed throughout development in regions undergoing patterning and morphogenesis and that are known to affect skeletal development. In particular, the exogenous administration of RA can alter Hox gene expression during concomitant homeotic transformations of axial segments (Kessel, 1992; Kessel and Gruss, 1991). Such transformations are manifested as mandibular hypoplasia, cervical ribs, or thirteenth ribs. In extreme cases, RA can produce caudal agenesis (Padmanabhan, 1998). The genetic knockout of particular RA receptors or dehydrogenases can lead to regional skeletal abnormalities (Cui et al., 2003; Niederreither et al., 2002), whereas the knockout of RA-metabolizing enzymes such as CYP26A1 causes more global skeletal dysmorphogenesis (Sakai et al., 2001). Sclerotomal regional identity is determined very early during development (Fomenou et al., 2005). Heterotopic segmental plate grafts between chick and quail have shown that, when cervical regions are transplanted into thoracic regions, ribs and scapula do not develop (Christ, 1978). RA is produced by cells of Hensen's node, and recent work has begun to link RA with the somitic oscillator and segment identity (Diez et al., 2003).

III. CRANIOFACIAL SKELETON DEVELOPMENT

Skeletogenesis of the skull is highly variable among species, it involves the exact coordinated interaction of several tissue types, and it is dominated by the differentiation of cranial neural crest cells, which is in contrast with the embryonic origins of the axial and appendicular skeleton. The study of craniofacial development thus presents new challenges. Nevertheless, the face is a defining feature of an individual's identity, and craniofacial abnormalities can have profound sociologic and physiologic consequences. Therefore, the skeletogenesis of the head and face is of intense research interest.

Several major subdivisions of the bones that comprise the skull are recognized: (1) the viscerocranium, which is the skeleton of the face and pharynx; (2) the cartilaginous neurocranium, which forms the base of the skull; and (3) the membranous neurocranium, which includes the bones that form the cranial vault. Elements of the viscerocranium can be further subdivided into cartilaginous and membranous categories. The subdivisions of viscerocranium and neurocranium are based on the process of bone formation that each undergoes.

A. Neural Crest

The principal cellular contributors to the craniofacial skeleton are the neural crest cells. Neural crest cells are a heterogeneous, multipotential group of cells that form during the differentiation of the neural plate from the ectoderm (see Chapter 26). They arise from the border of these two tissues at the dorsal edge of the neural folds along the entire length of the neural tube. These cells can contribute to a wide variety of tissues, including cranial nerves and meninges, dermis, muscle, connective tissues, and glands. Their fate varies on the basis of intrinsic gene expression and the extrinsic inductive signals that they receive during migration and at the site of destination (Le Dourain et al., 2004).

The contribution of neural crest of a certain origin to particular bones is still under investigation. *In vivo* studies involving techniques of laser ablation and the generation of chicken–quail chimeras have revealed the migratory pattern and regional existence of the neural crest cells in the bones of the head and face. However, the contribution of neural crest to cranial facial bones was only recently clarified using the Cre-lox transgenic technique that labeled neural crest cells with lacZ using the WNT-1 promotor, a factor that has been shown to be necessary and sufficient for neural crest induction (Jiang et al., 2000). With the exception of the parietal bones, all head and neck bones were strongly labeled with lacZ, thus indicating a strong or dominant contribution by the neural crest. In summary, neural crest cells contribute to the craniofacial skeleton to varying degrees and through different skeletogenic processes (Wilkie and Moriss-Kay, 2001). The neural crest cells that populate the pharyngeal arches and the cephalic paraxial mesoderm in the region of the prechordal plate and occipital somites contribute to the cartilaginous viscerocranium and the cartilaginous neurocranium via endochondral ossification, which was described previously. Neural crest cells that populate the mesenchyme that surrounds the dorsal half of the brain form the membranous neurocranium, and those that invade the first branchial arch form the membranous viscerocranium or facial bones. Both of these cranial bone sets are formed by intramembranous ossification, which is a process that is distinct from endochondral ossification.

B. Intramembranous Ossification

The membranous neurocranium consists of bones that form the cranial vault. These include the frontal and parietal bones (Figure 39.10, A and B), which form from the frontal and parietal eminences (the embryonic thickenings of the cephalic mesoderm in the prosencephalic and mesencephalic regions).

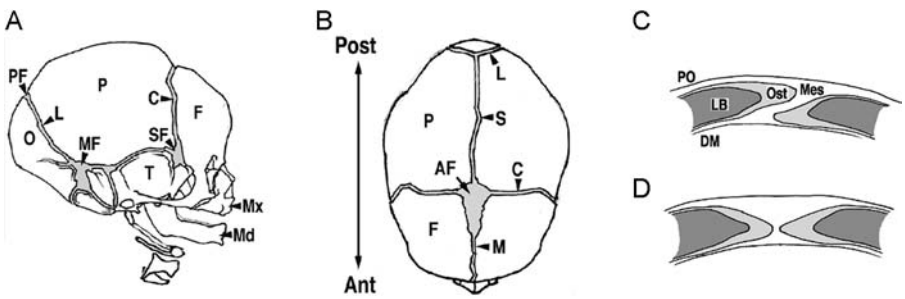

FIGURE 39.10 **Bones and sutures of the neurocranium.** Bones of the neonatal skull, **A,** laterally and **B,** dorsally. The neurocranium is composed of the frontal *(F)* and parietal *(P)* bones separated by the coronal *(C)*, sagittal *(S)*, and metopial *(M)* sutures that join at the anterior fontanelle *(AF)*. The neurocranium abuts the maxillary *(Mx)*, sphenoid (not shown), and temporal *(T)* bones of the viscerocranium, separated by the sphenoidal fontanelle *(SF)* and the occipital bone *(O)* of the chondrocranium, separated by the lambdoid suture *(L)* and the mastoid fontanelle *(MF)*. *Md,* Mandible. Illustrations of the two kinds of sutures, **C,** overlapping and **D,** abutting, showing the basic relationship between two lamellar bones *(LB)* with osteogenic fronts *(Ost)* separated by undifferentiated mesenchyme *(Mes)* in close relationship with the underlying dura mater *(DM)* and the overlying periosteum *(PO)*. (Adapted from Sadler TW: *Langman's medical embryology,* 7th ed, Baltimore, Md, 1995, Williams and Wilkins.)

These two types of eminences are actually of differing tissue origins: the frontal is of neural crest origin, and the parietal is of cephalic paraxial mesoderm. Despite their differences in origin, they both produce bone by an alternative skeletogenic process called *intramembranous ossification*.

Although endochondral ossification requires a cartilaginous model before bone deposition, intramembranous ossification is characterized by mesenchymal cells that condense and directly form osteogenic nodules (Ornitz and Marie, 2002). These nodules form in close association with blood vessels of the dura mater (in cranial vault development) or of other membranes, such as the perichondrial sheath of Meckel's cartilage (in mandibular development). In fact, the dura mater and the overlying perichondrium are essential for calvarial morphogenesis, postnatal suture fusion, and osseous repair of calvarial defects. In these nodules, proteoglycan-rich chondroid is produced, and it is quickly calcified to osteoid (Lengele et al., 1996). Cells that are encased in this matrix become osteoblasts, whereas those that surround the nodules become the periosteum. The inner layer of the periosteum gives rise to a progenitor population that undergoes osteogenesis and that forms layers of bone or lamellae that characterize membranous bones (Figure 39.11).

Macroscopically, this process advances from the initial nodules through the formation of long spicules of bone that fuse with one another to form plates. The bones of the cranial vault expand to abut one another during development, but they do not fuse. The juncture between cranial vault bones becomes a functional structure called a *suture,* which coordinates the growth of the skull with the underlying brain. The suture is an anatomically simple structure that is composed of two apposing plates in juxtaposing or overlapping configuration that are separated by a narrow space that is analogous to a growth plate (see Figure 39.10, C). The suture contains regions of

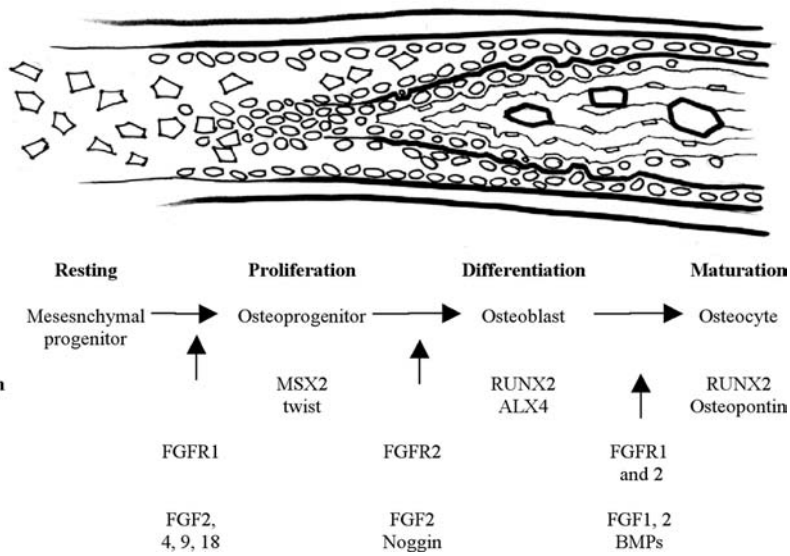

FIGURE 39.11 Microanatomy of the suture and molecular pathways regulating suture growth and osteogenesis. A schematic diagram of suture differentiation zones showing selected transcription factors expressed in the cells of the suture and signaling molecules emitted by the dura mater influencing the overlying mesenchyme. (Adapted from Ornitz and Marie, 2002.)

undifferentiated mesenchyme, rapidly dividing osteogenic stem cells, differentiated osteoblasts producing osteoid, and mature osteoblasts that are encased in a bony matrix (see Figure 39.11). The growth and timing of suture closure is carefully controlled by its interaction with the underlying dura mater in a manner that is analogous to the perichondrium/periosteum of endochondral ossification.

The molecular mechanisms of intramembranous ossification and suture growth are not as well characterized as endochondral ossification and growth plate growth. The direct ossification (or the lack of chondrogenic intermediate) during the intramembranous process has been clearly shown in RUNX2 knockout mouse. RUNX2 is a master regulator of osteoblast-differentiation–regulating genes such as osteocalcin, osteopontin, bone sialoprotein, collagens, alkaline phosphatase, vitamin D receptor, osteoprotegerin, TGF-β receptors, and others (Stein et al., 2004). In accordance with this, investigators found that RUNX2 knockout homozygotes produce a cartilaginous skeleton without any indication of osteogenesis (Otto et al., 1997). In addition, the membranous neurocranium and the medial clavicle are completely absent. The heterozygotic phenotype is reminiscent of the human condition called cleidocranial dysplasia, which is caused by a mutation of the human RUNX2 gene (Mundlos et al., 1997). This is one of the few mutations that reveal a genuine error in the intramembranous mechanism itself.

Craniosynostosis occurs in 1 in every 2500 live births, and it is caused by the premature fusion of one or more sutures. Errors in suture closure are attributed to imbalances in the proliferative and differentiation phases of suture growth. Studies of the molecular genetics behind craniosynostosis have provided information about important players during the various phases of suture development, with activating mutations in FGFR-2 being the most frequently implicated (Table 39.2; Marie et al., 2005). However, other FGF members are expressed during intramembranous ossification in the suture, and recent work has shown the involvement of other molecules that begin to build a pathway of osteogenesis in the suture (Nie et al., 2006).

Loose, resting mesenchymal cells within the suture are poorly characterized, but it is known that these cells express FGFR-1 and that they are under the influence of several FGFs, including FGF2, -4, -9 and -18 (see Figure 39.11). Markers of these cells in particular are not known, but it is known that the forced expression of Hox genes (e.g., Hoxa-2, which is required for visceral cranial neural crest osteogenesis) actually inhibits suture development in the neurocranium (Creuzet et al., 2002; Couly et al., 2002). Proliferative mesenchymal cells in the suture express Twist and muscle segment homeobox 1 (MSX1), which are transcription factors that maintain cells in an undifferentiated state in several developmental contexts (Takahashi et al., 2003; Soo et al., 2002; Ishii et al., 2003). FGF-2 plays a dominant role at this stage. A switch to FGFR-1 is important for initiating differentiation and the expression of RUNX2 and aristaless-like 4 (ALX4) by downregulating the expression of twist and MSX2 (Ishii et al., 2005). Both MSX2 and Twist mutant mice reveal interesting features in intramembranous ossification. Dominant-negative mutations in MSX2 accelerate the differentiation of the sutural mesenchyme, and this results in Boston-type craniosynostosis. Alternatively, activating mutations of MSX2 prevent differentiation that leads to parietal foramina (an open suture; Winograd et al., 1997). This illustrates the delicate balance between mesenchymal proliferation and the differentiation

TABLE 39.2 Selected Fibroblast Growth Factor Receptor, Msx2, and Twist Mutations and Their Associated Human Syndromes

Gene	Mutation	Syndrome	Phenotype
FGFR2	C342R	Pfeiffer Crouzon Jackson–Weiss	Sagittal synostosis or cloverleaf skull, duplication of thumbs and big toe Variable cranial synostosis, hypertelorism, beaked nose, hypoplastic maxilla, and mandibular prognathism with no limb abnormalities Variable cranial synostosis, midfacial hypoplasia, and foot anomalies
	P253R	Apert	Variable cranial synostosis, midfacial hypoplasia, hand and foot syndactyly and variable fusion of hand, foot, and vertebral fusions
	S252W or F	Apert	
	S252L	Normal (mild Crouzon)	No cranial synostosis with variable syndactyly
FGF3	P250R	Muenke	Coronal synostosis, variable syndactyly and brachydactyly of the hand
FGFR1	P252R	Pfeiffer	Sagittal synostosis or cloverleaf skull, duplication of thumbs and big toe
Twist	S123stop	Saethre–Chotzen	Asymmetric cranial synostosis, widely spaced eyes, beaked nose, syndactyly and brachydactyly of the hand
	416ins21 (insKIIPTLP)	Saethre–Chotzen	
Msx2	P148H	Boston craniosynostosis	Cloverleaf skull, supraorbital recession

that governs suture development (Liu et al., 1999). The importance of Twist is seen in cases of Saethre–Chotzen syndrome, in which an inactivating mutation in Twist leads to early suture fusion. Recently, the switch to mesenchymal differentiation in the suture was shown to be enabled by the activation of the BMP pathway (Kanzler et al., 2000), and this resulted in changed Twist expression. Noggin, which is an endogenous inhibitor of BMPs, is expressed in open sutures, and its downregulation is associated with suture closure that is concomitant with a decrease in Twist expression. Importantly, activating mutations in FGFR-2 that are known to cause early suture osteogenesis and closure has been shown to decrease Noggin expression.

Having described a generalized sequence of suture growth regulation, the incidence of particular craniosynostotic patterns with specific genetic mutations argues that there are differences among sutures. Two specific knockout mice illustrate the point. EphrinB1 knockout mice have premature metopic suture closure (Twigg et al., 2004), whereas axin2 knockout mice are characterized by premature coronal suture closure (Yu et al., 2005). These two sutures regulate growth between the neurocranial bones of cranial paraxial mesodermal or neural crest origin, respectively. Thus, the specific molecular regulatory mechanisms of osteogenesis in different sutures can vary on the basis of the origin of the constituent cells.

Osteogenic differentiation is thought to occur by similar means in both endochondral and intramembranous ossification, because RUNX2 is required for both processes. In fact, the observation that limb abnormalities are associated with many craniosynostoses suggests that craniofacial and limb

development share some mechanistic processes, such as the requirement for RUNX2 in osteogenesis and the involvement of the FGF signaling pathways in bone growth. Another similarity is the activity of the parathyroid hormone/IGF-1 axis involved in osteogenesis and calcium homeostasis. However, the activation of this pathway is clearly different between the intramembranous and endochondral ossification mechanisms (Suda et al., 2001). In endochondral ossification, Ihh regulates the transition from proliferative to hypertrophic chondrocytes and induces the expression of PTHrP signaling components. Without a cartilaginous intermediate, the PTHrP pathway is expressed during intramembranous ossification in a manner that is independent of Ihh. The means of this induction is still unknown, thus revealing subtle differences in the mechanisms of osteogenesis in the long bones and the calvaria that are yet to be elucidated.

Techniques of experimental embryology, such as tissue transplants (e.g., chick–quail chimeras) and organ explant cultures, play an important role in the formation of the principles of development from a morphologic perspective, thereby suggesting cellular and molecular behaviors and interactions that are required to produce the changes observed. Genetic analyses have revealed the link between phenotype and genotype, and, when these analyses are applied to developmental problems, they shed light on the specific cellular and molecular pathways that characterize early ontogeny. These applications include characterizations of spontaneous mutants through positional cloning (e.g., Pudgy), knockouts (RUNX2), and transgenesis (Wnt-1–lacZ). A detailed understanding of embryology is required for the proper interpretation of these experiments. As tissue-specific expression approaches (e.g., Cre-Lox and other inducible systems) become more refined and widespread, it will be possible to manipulate in utero specific cell and tissue interactions that are otherwise inaccessible or previously unidentified. As discussed previously, these techniques are beginning to clarify fundamental mechanisms of skeletogenesis. The advent of genomics expands the experimental approaches to skeletal development, thus enabling the identification and analysis of candidate genes and their function(s) and establishing the different "transcriptosomes" responsible for the developmental processes that are critical to skeletogenesis. Information obtained from these studies should provide insights into disease causes, phenotype variability, wound healing, and the mechanisms of oncology.

SUMMARY

- Bones and connective tissues of the body are derived from two embryologic sources: the mesoderm and the neural crest. The appendicular skeleton arises from the lateral plate mesoderm, the axial skeleton arises from the paraxial mesoderm, and the craniofacial skeleton arises from a combination of the cephalic paraxial mesoderm and neural crest cells.
- Bones are formed through one of two processes:
  —*Endochondral ossification* involves the formation of a cartilaginous model, which is subsequently replaced by bone.
  —*Intramembranous ossification* is characterized by the direct differentiation of mesenchymal cells to osteogenic tissue.
- Bone growth is regulated by specific, analogous growth zones: the growth plate in the long bones and the sutures between cranial bones. The FGF

signaling system plays a critical role in the regulation of these growth zones. Mutations in different FGF receptors cause achondroplasias and craniosynostoses.

- The replacement of cartilage by osteoblasts is dependent on the prior mineralization of the cartilage matrix and vascular invasion. The activities of the hypertrophic chondrocytes and of the osteoblasts are regulated by RUNX2.
- Osteoclasts are of hemopoietic origin (i.e., monocytes), and, with osteoblasts, they remodel bone throughout an individual's lifetime to adapt to mechanical demands.
- Joints are formed via the bifurcation or apposition of chondrogenic models. WNT-14 and the activity of β-catenin are central regulators of joint formation. The cellular origin of articular cartilage is unknown.
- Somites are formed from the segmental plate through the interaction of a cell-autonomous oscillator and a morphogenetic wave that coordinates somite boundary formation via the Notch–Delta juxtacrine system and epithelialization via the WNT paracrine system and cadherin activity. The dysregulation of this process leads to congenital scoliosis.
- The axial skeleton is derived from the ventromedial sclerotome of somites. The differentiation of the somite is regulated by paracrine factors from surrounding tissues, and the sclerotome in particular is induced by Shh.
- Tendons and ligaments develop from the interaction of mesodermal mesenchyme with myoblasts.
- The nucleus pulposus of the intervertebral disc is initially derived from the notochord. The gradual loss of "embryonic cells" from the disc may be related to disc degeneration and back pain.
- The craniofacial skeleton is derived from cells of different origins, and it forms through either intramembranous or endochondral ossification. The constituent cells' origin and local environment determine the specific mechanisms of osteogenesis.

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GLOSSARY

Endochondral ossification
A mechanism of bone formation in which a cartilage model is replaced with ossified tissue that is produced by invading osteoblasts.

Growth plate
The area of developing tissue between the diaphysis and the epiphysis and that is responsible for the longitudinal growth of bones. The suture, which is an analogous structure between the bones of the neurocranium, coordinates skull growth with brain development.

Hypertrophy
An increase in bulk without a concomitant multiplication of parts.

Interzone
A region of nonchondrogenic cells that forms at the site of a future joint.

Intramembranous ossification
A mechanism of bone formation in which ossified tissue is produced directly from condensed mesenchyme lying in close association with a membranous structure.

Somite
An embryonic tissue that patterns and maintains the metamerism of axial tissues.

Resegmentation
The process by which the sclerotomes of neighboring somites reorganize to form vertebral bodies that span the space between the dermomyotomal derivatives of the same somites.

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Online Mendelian Inheritance in Man: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM

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CRANIOFACIAL DEVELOPMENT/INTRAMEMBRANOUS OSSIFICATION

Marie PJ, Coffin JD, Hurley MM: FGF and FGFR signaling in chondroplasia and craniosynostosis, *J Cell Biochem* 95:888–896, 2005.
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VI

ENDODERMAL ORGANS

40

PATTERNING THE EMBRYONIC ENDODERM INTO PRESUMPTIVE ORGAN DOMAINS

BILLIE A. MOORE-SCOTT and JAMES M. WELLS

Division of Developmental Biology, Cincinnati Children's Hospital Research Foundation and University of Cincinnati College of Medicine, Cincinnati, OH

INTRODUCTION

The process of gastrulation subdivides the embryonic epiblast into the three primary germ layers: the ectoderm, the mesoderm, and the endoderm. During organogenesis, the endoderm germ layer contributes to many vital organs, including the liver, the pancreas, the thymus, and the thyroid, and it forms the epithelial lining of the gastrointestinal and respiratory tracts (Figures 40.1 and 40.4; reviewed by Wells and Melton, 1999). The endoderm cells of the postgastrulation vertebrate embryo are largely unspecified, and they become regionalized along the anterior–posterior (AP) and dorsal–ventral (DV) axes during the process of the formation of a primitive gut tube. The process during which endoderm cells obtain positional identity along the AP and DV axes is called *patterning,* which is a fundamental event that is necessary for directing endoderm cells into specific organ lineages. In addition, the endoderm itself acts as a source of signals that regulate the proper development of mesoderm and ectoderm organs such as the anterior central nervous system and the heart. Therefore, failure to appropriately pattern the endoderm can have a broad impact on the developmental outcome of the entire embryo.

Several vertebrate animal model systems have greatly contributed to our understanding of early endoderm organogenesis, including Xenopus, zebrafish, chicken, quail, and mouse. It is remarkable that many aspects of early endoderm organogenesis are conserved across vertebrate species. For example, within a few days after gastrulation in amphibians, fish, birds, and mammals, unspecified endoderm cells form a primitive gut tube with distinct AP and DV domains that predict the formation of organ primordia. Furthermore, there is increasing evidence that signals and responding target genes

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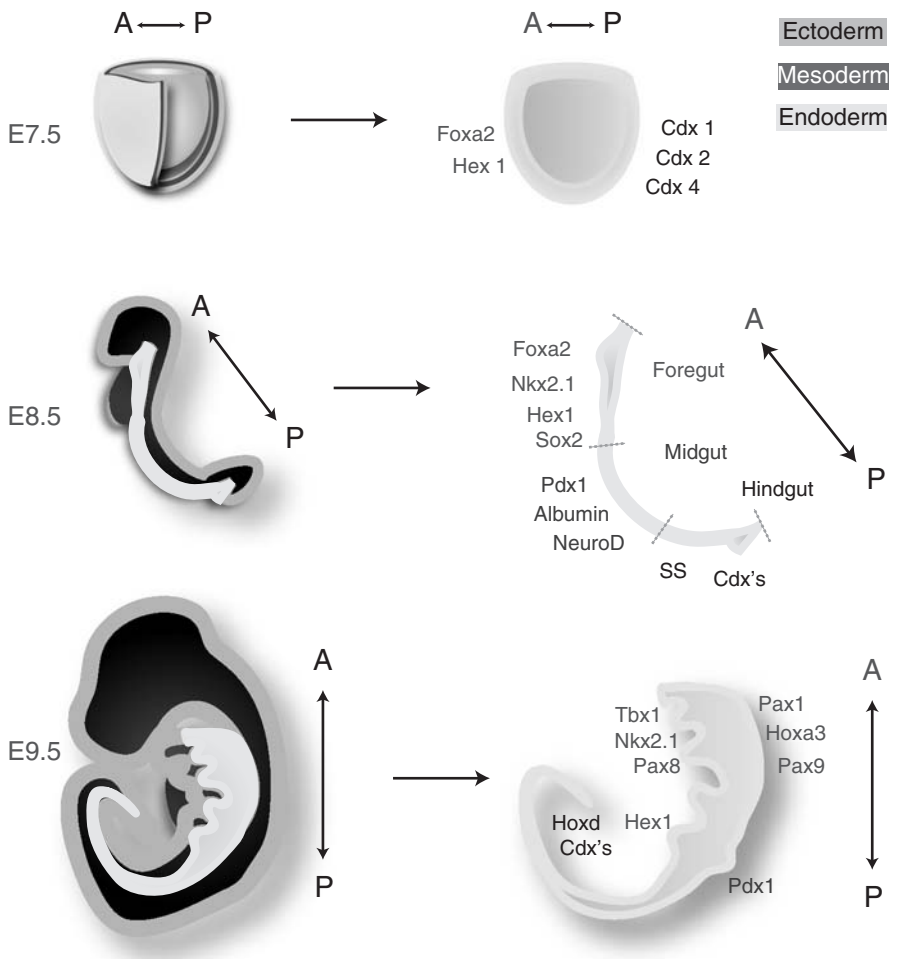

FIGURE 40.1 Early stages of endoderm organogenesis in the mouse. The left panels show a schematic of mouse embryos, and the right panels highlight the endoderm and genes that are regionally expressed along the anterior–posterior and dorsal–ventral axes. After gastrulation, which is complete 7.5 days after fertilization in the mouse, the endoderm is the outermost layer of cells *(yellow)* that surround the embryo. The middle layer is the mesoderm *(red)*, and the inner layer is the ectoderm *(blue)*. After one day of embryonic development (i.e., on embryonic day 8.5), morphogenetic movements at the anterior and posterior initiate tube formation and start the process of internalizing the endoderm. These processes result in the formation of a primitive gut tube with foregut and hindgut tubes. The middle section of the primitive gut tube (midgut) remains open at this stage. By 10.5 days of embryonic development, the formation and internalization of the primitive gut tube is complete, and organ primordia for the lungs, liver, and pancreas are morphologically evident. (See color insert.)

that direct the formation of a patterned gut tube are largely conserved across vertebrate species. In this chapter, we will discuss the morphogenetic and molecular processes that are involved in subdividing the endoderm into functional presumptive organ domains along the AP axis, with a focus on studies in the chick and mouse. These early stages of endoderm organogenesis are critical for the proper development of the gastrointestinal and respiratory tracts and for the ontogeny of the associated vital organs.

The formation and patterning of the primitive gut tube is a dynamic morphogenetic process that involves cell migration and tissue remodeling. For simplicity, we have subdivided the gut tube into the foregut, the midgut, and the hindgut (Figure 40.1). The anterior segment of the gut tube—the foregut—forms first and comes from the endoderm extending from the most anterior portion of the embryo (headfold in the postgastrula embryo, embryonic day 7.5 to 8 in the mouse) to just below the cardiac mesoderm. The foregut endoderm contributes to a remarkable number and diversity of organs, including the taste buds, the inner ear, the thyroid, the thymus, the esophagus, the trachea, the lungs, the liver, and the ventral pancreas. The hindgut forms shortly after the foregut, and it is derived from the most posterior region of the gastrula stage endoderm overlying the primitive streak. The hindgut contributes to the small and large intestines as well as to the bladder and the urogenital tract. For the purposes of this chapter, we will refer to the open portion of the gut tube during these early stages of gut tube morphogenesis as the *midgut*. This is the last region of the endoderm to form a tube, and it is comparatively larger than the traditionally defined midgut, which is based on anatomic descriptions of the gut of later-stage embryos. The midgut endoderm as defined at this stage contributes to part of the stomach, to the dorsal pancreas, and to the anterior portions of the small intestine, such as the duodenum.

I. FATE MAP OF THE EMBRYONIC ENDODERM

The endoderm organs that develop from the foregut, midgut, and hindgut become morphologically apparent within 2 days of gut tube formation (embryonic day 10.5 in the mouse). To better understand the early stages of endoderm organogenesis, we will discuss in this section the embryonic origins of the different AP and DV domains of the gut tube. Numerous studies have begun to identify the cellular origins of endoderm organ domains using a method called *lineage tracing* or *fate mapping*. Lineage tracing is a method during which cells are labeled and followed from an early progenitor stage to a more mature stage of development (Figure 40.2). Thus, an endoderm cell labeled at the late gastrula stage can be traced to specific organ domains as embryonic development progresses. Cell lineage

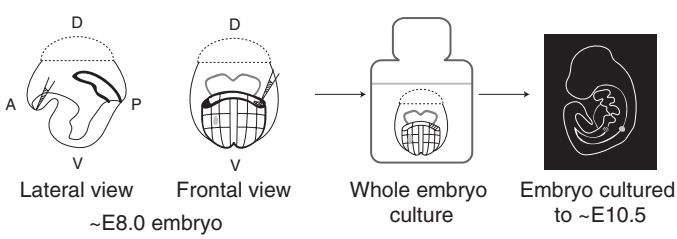

FIGURE 40.2 **Labeling endoderm cells for lineage analysis and generating fate maps.** Cells can be labeled by injecting vital dyes *(striped; DiI)* or by expressing fluorescent proteins *(gray)*. Labeled endoderm cells can be tracked through subsequent stages of development. The two left panels show a lateral and frontal view of an early somite stage embryo (~3 somites, e8 mouse). Embryos can be cultured *in vitro*, and the positions of the labeled cells can be determined after 1 to 3 days of development. This approach has been used to identify which regions of the early endoderm give rise to specific gut tube and organ domains and to generate fate maps.

analyses performed in the mouse, chick, and frog have mapped the movement of gastrulation-stage endoderm cells to broad domains of the developing gut tube and early organ primordia (Chalmers and Slack, 2000; Kimura et al., 2006; Lawson and Schoenwolf, 2003; Lawson et al., 1986; Rosenquist, 1971; Tam et al., 2004). These studies suggest that many aspects of early endoderm patterning and gut tube formation are remarkably conserved across vertebrate species. Later in this chapter, we will compare the fate-mapping studies performed in two model organisms: the chick and the mouse.

A. Mapping Endoderm Cell Lineage During Gut Tube Morphogenesis: From Gastrula Stages to the Early Somite Stages in the Chick and the Mouse

1. Endoderm Fate Maps at the Gastrula Stages

In the mouse and the chick, endoderm cells form during gastrulation and migrate out of the primitive streak to integrate into the outer layer of cells, thereby forming the endoderm germ layer (see Chapter 14). Gastrulation in the mouse occurs between embryonic days 6 and 7.5; in the chick, this occurs between 7 and 19 hours of development (Hamburger and Hamilton [HH] stages 2–5). Cell lineage analyses of early to mid-gastrula embryos of both species suggest that the time at which endoderm cells exit the primitive streak influences where they end up along the AP axis at the end of gastrulation. For example, during the early mouse gastrula (embryonic day 6–6.5), the first endoderm cells to exit the primitive streak preferentially end up in the anterior endoderm overlying the anterior neural plate and trunk at the end of gastrulation (embryonic day 7.5; Lawson and Pedersen, 1987). During the mid- to late gastrula stages (embryonic day 7–7.5), endoderm cells exiting the primitive streak often colonize more posterior domains and remain adjacent to the node and the primitive streak. These fate-mapping studies suggest that the anterior axial endoderm cells are the first endoderm cells to form during gastrulation in the mouse, whereas posterior endoderm forms slightly later.

Studies in the chick have added to our understanding of when different domains of endoderm are formed, and they have raised some interesting questions (Kimura et al., 2006; Lawson and Schoenwolf, 2003). One experiment that traced the lineage of axial/midline endoderm cells reached a similar conclusion to the studies in the mouse: that anterior endoderm forms first and is followed by more posterior trunk endoderm (Lawson and Schoenwolf, 2003). However, another study suggested that posterior/lateral endoderm is the first to incorporate into the hypoblast layer during the early gastrula stage (Kimura et al., 2006). These different studies could suggest that midline and lateral endoderm arise from different populations of endoderm precursors, and they emphasize how lineage-tracing experiments continue to reveal new and surprising aspects of endoderm development. For example, one study revealed a population of endoderm cells that exits the epiblast during gastrulation and migrate for several hours before inserting into the endoderm germ layer during the late gastrula stage (Kimura et al., 2006).

B. Mapping Late Gastrula Stage Endoderm to the Developing Gut Tube

The studies described previously indicate that the late gastrula endoderm (embryonic day 7.5 in the mouse; HH stages 4 and 5 in the chick) may have distinct AP and lateral domains. This section will describe how these domains

within a two-dimensional sheet of endoderm will give rise to the AP and DV domains of a three-dimensional gut tube (Wells and Melton, 1999). Figure 40.3 compares the chick and mouse endoderm fate maps and illustrates the relationships among the domains of the gastrula stage endoderm (embryonic day 7.5 in the mouse; HH stages 4 and 5 in the chick), the developing gut tube at early somite stages (embryonic day 8.5 in the mouse; HH stage 10 in the chick), and the gut tube at a stage when organ primordia start to become morphologically apparent (embryonic day 9.5 in the mouse; HH stage 17 in the chick). Figure 40.3 is a composite of several cell lineage studies that have begun to reveal the complex

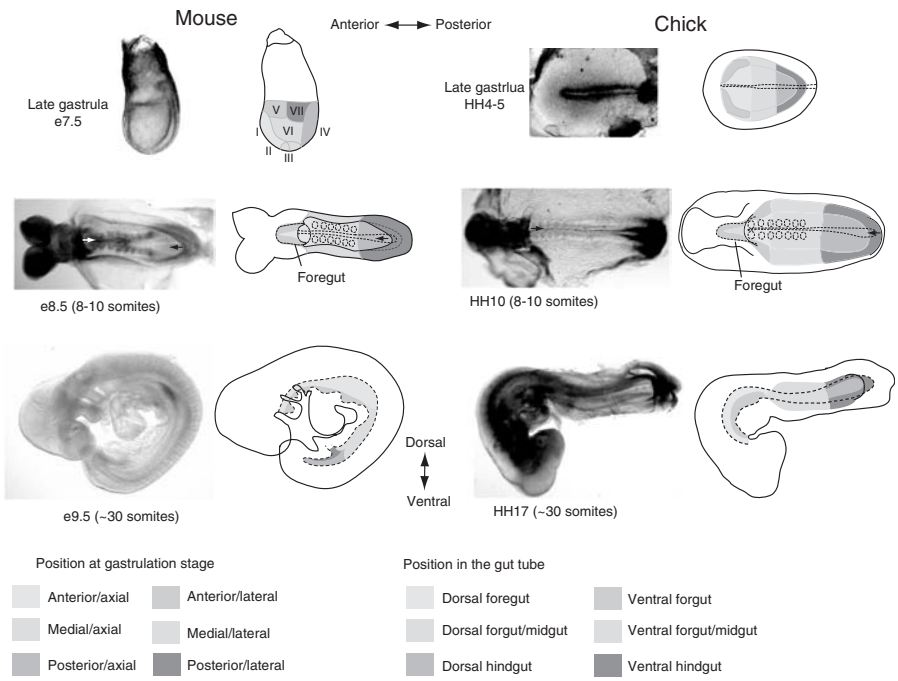

FIGURE 40.3 **Fate map of the endoderm and gut tube in the mouse and the chick.** In the top panels, late gastrula stage mouse (lateral view) and chick (ventral view, endoderm facing up) embryos are shown. The *dotted line* in the chick shows the developing notochord. The roman numerals (I–VII) indicate the domains identified in the mouse fate-mapping studies (Lawson et al., 1986; Tam et al., 2004). In the middle panels, a ventral view of 8- to 10-somite stage embryos (embryonic day 8.5 in the mouse; Hamburger and Hamilton stage 10 in the chick) is shown in which the foregut has started to form and to be internalized. The *arrows* indicate the folding/migration direction of the foregut and the hindgut. The *dotted circles* in the middle panels are somites, and the *dotted line* along the midline is the developing notochord. The dotted line in the anterior outlines the developing foregut. In the bottom panels, a lateral view of mouse (embryonic day 9.5; blue-staining cells are *Pdx1-LacZ* expression) and chick (Hamburger and Hamilton stage 17) embryos at the approximately 30-somite stage is shown. At this stage, the endoderm has been internalized and folded into a gut tube, with the exception of the hindgut in the chick, which has not fully formed at this stage. The colored domains of the gastrula-stage embryos roughly correspond with the equivalent-colored domains of the later-stage embryos. The axial endoderm of the gastrula embryo (domains I, II, III, and IV) contributes to both the dorsal and the ventral gut tubes, although only domains I and IV contribute ventrally. The lateral domains (V, VI, and VII) fold over to principally contribute to the ventral gut tube. For all images, the anterior is left and the posterior is right. In the mouse, the fate map of the foregut between embryonic days 8.5 and 10 has been well studied (Tremblay and Zaret, 2005). The posterior domains at these stages have not been well studied, and the domains in this figure are extrapolated from the earlier-stage fate map. (The *Pdx1-LacZ* mice were from Chris Wright of Vanderbilt University. See color insert.)

morphogenetic processes involved in the formation of the gut tube (Kimura et al., 2006; Lawson et al., 1986; Rosenquist, 1971; Tam et al., 2004; Tremblay and Zaret, 2005).

In the mouse, cell lineage experiments performed more than 20 years ago and more recent studies have resulted in a detailed fate map of the late gastrula stage (embryonic day 7.5) of the mouse endoderm (Lawson et al., 1986; Tam et al., 2004). These studies identified regions of endoderm on embryonic day 7.5 that will contribute to the distinct AP and DV domains of the developing gut tube (see Figure 40.3). One recent study distinguished between endoderm cells located along the midline versus those located more laterally. Midline endoderm cells tend to remain along the midline in both the ventral and dorsal regions of the gut tube. By contrast, endoderm cells that are found laterally in the embryonic day 7.5 endoderm preferentially end up in the ventral portions of the gut. These fate-mapping studies concluded that the most anterior midline endoderm cells overlying the neural plate (see Figure 40.3; region I) as well as the anterior lateral endoderm (region V) both contribute to the ventral foregut, which gives rise to the liver, the ventral pancreas, and the lungs (Lawson et al., 1986). Axial endoderm cells just anterior to the node (region II) contribute to the dorsal foregut, which forms the dorsal component of the stomach, the pancreas, and the duodenum, whereas lateral endoderm from region VI contributes to the ventral midgut. Axial endoderm overlying the node and the anterior primitive streak (region III) contributes to the dorsal midgut and hindgut, eventually contributing to the small intestines. The most posterior endoderm overlying the primitive streak (see Figure 40.3; regions IV and VII) contributes to both the dorsal and ventral portions of the hindgut, which contribute to the posterior intestinal derivatives and the urogenital tract.

Again, studies suggest that the chick fate map roughly corresponds with that of the mouse (Kimura et al., 2006; Lawson and Schoenwolf, 2003; Rosenquist, 1971): the anterior axial and lateral domains of HH stage 5 endoderm contribute to the ventral foregut at HH stage 10, the endoderm overlying the middle primitive streak contributes to dorsal midgut and hindgut, and the most posterior endoderm over the primitive streak contributes to the ventral portions of the midgut and hindgut. The combined evidence suggests that the presumptive AP and DV regions of the gut tube are established as early as the gastrula stage, before gut tube morphogenesis (see Figure 40.3). Furthermore, the lineage tracing experiments demonstrate that gut tube morphogenesis involves the folding and migration of the anterior/lateral endoderm to make the foregut and of the posterior/lateral endoderm to make the hindgut. The folding/migration of the foregut continues toward the posterior, and similar expansion of the hindgut continues toward the anterior, thereby resulting in the closure of the primitive gut tube.

C. Regions of the Foregut That Give Rise to Developing Organ Primordia

Less is known about the domains of the early gut tube that give rise to developing the mid-gestation–stage organ primordia. However, a study describing lineage tracing experiments performed on early somite-stage mouse embryos identified domains in the developing foregut that contribute to specific foregut derivatives, including the liver and the ventral pancreas (Tremblay and Zaret, 2005). In these studies, foregut endoderm cells were labeled on embryonic day

8 to 8.5 (1–7 somites), and their descendants were analyzed at the early liver bud stage (embryonic day 9.5). One medial and two lateral domains of foregut endoderm were found to contribute to the liver bud. In addition, cells of the medial domain at the lip of the developing foregut gave rise to descendants all along the ventral midline, whereas the lateral domains contribute to specific ventral regions such as the liver. This study supports the idea that medial and lateral endoderm cells continue to migrate independently and then later converge during gut tube morphogenesis during the formation of the liver and other ventral foregut derivatives.

The experiments described previously as well as studies in other organisms, including Xenopus and zebrafish, highlight the complex morphogenetic processes that occur during the formation and patterning of the primitive gut tube. The later sections discuss specific signaling pathways and target genes that functionally regulate these early stages of endoderm organogenesis.

II. GENE EXPRESSION DOMAINS PREDICT AND DETERMINE ENDODERM ORGAN PRIMORDIA

Genes have been identified that are expressed in discrete AP and DV domains during the development of the gut tube. These markers are invaluable for the understanding of how endoderm is patterned at the molecular level. Many of these marker genes both predict where the endoderm organ primordia will form and play a functional role in establishing organ boundaries (Figure 40.4). In this section, we will discuss molecular studies that describe how patterns are established in the early endoderm and refined in the primitive gut tube, and we will also address the role of several of these genes during endoderm organogenesis.

At the end of gastrulation in Xenopus, chick, and mouse, most reported gene expression patterns are in broad overlapping domains along the AP axis. For example, in all three species, *Hematopoietically expressed homeobox1 (Hex1), orthodenticle homologue 2 (Otx2*), and *forkhead box A2 (Foxa2)* are expressed along the anterior half of the embryo, whereas the *Caudal type homeobox* genes *Cdx1, Cdx2,* and *Cdx4* (*CdxA* and *CdxC* in the chick) are broadly expressed in the posterior half (see Figure 40.1; Ang et al., 1993; Chapman et al., 2002; Frumkin et al., 1993; Gamer and Wright, 1993; Jones et al., 1999; Northrop and Kimelman, 1994). The expression domains of these factors suggest that the endoderm is broadly patterned along the AP axis at this stage. Moreover, gene targeting experiments have demonstrated that *Hex1* is vital for anterior patterning as well as for subsequent thyroid and liver formation (Martinez Barbera et al., 2000), and the loss of *Cdx2* function results in posterior truncations around the time of gut tube formation (Chawengsaksophak et al., 2004). *Foxa2* is functionally important for the formation of the anterior primitive streak, and an endoderm-specific knockout of *Foxa2* and of the closely related factor *Foxa1* results in the failure to initiate liver development (Lee et al., 2005).

By the time that gut tube formation occurs (embryonic day 8.5 in the mouse; HH stage 10 in the chick), a complex pattern of gene expression emerges in the foregut *(Nkx2.1, Hex1,* and *Sox2)*, the posterior foregut (albumin), the midgut *(Pdx1)*, and the hindgut *(Cdx2* and *Cdx4; CdxA* and *CdxB* in the chick;

Figures 40.1 and 40.6; Chalmers et al., 2000; Gamer and Wright, 1993; Gittes and Rutter, 1992; Ohlsson et al., 1993; Zeng et al., 1998). The extensive patterning of the primitive gut tube suggests a high degree of gene regulation. Two well-studied examples of highly regulated genes are *Pdx1* and *Cdx*, which are part of the ParaHox cluster that arose from an ancestral ProtoHox cluster. The ParaHox cluster exhibits conserved spatial expression along the AP axis in divergent animal phyla, where *Cdx* expression defines the domain posterior to *Cdx* (the small and large intestines) and *Pdx1* expression defines the domain anterior to *Cdx* (the duodenum, the pancreas, and the caudal stomach). These transcription factors may play a role in maintaining their mutually exclusive expression domains. For example, in the chick, the misexpression of *Pdx1* in the posterior results in the repression of the *Cdx* genes, which suggests that *Pdx1* directly or indirectly restricts the anterior expression limit of *Cdx* genes (Grapin-Botton et al., 2001). In addition, the genetic ablation of *Pdx1* in the mouse results in arrested pancreatic development and abnormal epithelial development in the rostral duodenum (Grapin-Botton, 2005).

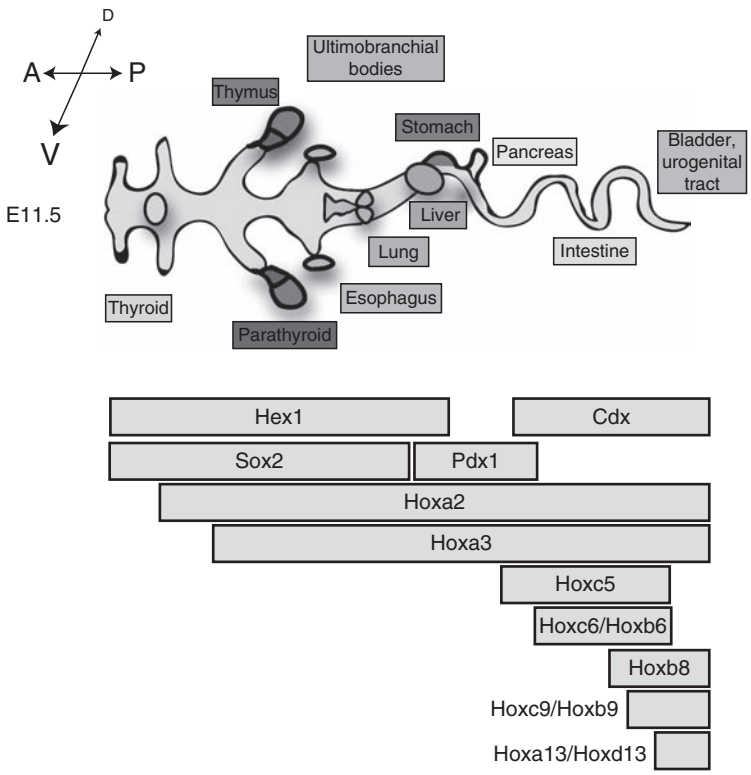

FIGURE 40.4 Overlapping expression domains of transcription factors predict the emergence of organ primordia along the anterior–posterior axis of the fetal gut tube. The upper schematic shows a ventral view of a fetal gut tube around the time of organ bud formation (embryonic day 10.5–11 in the mouse). Anterior is left and posterior is right. The lower panel indicates the relative anterior–posterior expression boundaries of several homeobox-containing transcription factors. *Hex1* and *Cdx* factors (*Cdx1, Cdx2,*and *Cdx4* in the mouse; *CdxA* and *CdxB* in the chick) are expressed in the gastrula-stage endoderm (embryonic day 7.5 in the mouse), whereas other genes, such as *Pdx1,* are first expressed later in the gut tube (embryonic day 8.5 in the mouse). The anterior and posterior expression limits of some of these factors are important for establishing organ domains. (The lower panel was adapted from Grapin-Botton, 2005. See color insert.)

By embryonic day 9.5, the gut tube has expanded both anteriorly and posteriorly. At this stage, the pattern in the anterior foregut becomes further refined as indicated by the restricted expression of several genes (see Figure 40.1). *Nkx2.1* is expressed in two domains, and this marks the presumptive thyroid and lung. *Pax1, Pax9, Tbx1, Hoxa3,* and *Pax8* are expressed in the presumptive pharyngeal domains, and the *Hoxd* genes mark the posterior pharyngeal endoderm (discussed later). By embryonic day 10.5 in the mouse, organ primordia for the thyroid, the lungs, the pancreas (both ventral and dorsal), and the liver begin to bud from these patterned domains. Subsequent stages of endoderm organ development are discussed in more detail in other chapters of this book.

A. Anterior–Posterior Patterning by Hox Genes

One mechanism by which ParaHox transcription factors are believed to control patterning is through the regulation of the Hox genes. Hox genes are key transcriptional regulators of embryonic patterning in chordates, and they are well known for their role in patterning mesoderm and ectoderm. Hox genes are also expressed in defined domains along the AP axis of the gut (Figure 40.4), and emerging evidence suggests that they are involved in the development of the primitive gut and its derivatives (Grapin-Botton, 2005). In the mouse, the Hox cluster consists of 39 genes in four linkage groups (A, B, C, and D), with 13 paralogous families (see Chapter 9). In both the mouse and the chick, the expression domains of Hox genes have been identified along the AP axis of the developing fetal gut (Grapin-Botton, 2005). In the pharyngeal region, *Hoxa2, Hoxa3,* and *Hoxa4* have overlapping patterns of expression with different anterior expression limits, thus defining the identity of the pharyngeal arches (Graham and Smith, 2001). *Hoxc5* is expressed in a broad domain between the caudal stomach and the anterior intestine whereas *Hoxc9, Hoxb8,* and *Hoxb9* are expressed in the most posterior portion of the fetal gut. *Hoxb6* and *Hoxc6* are expressed in restricted domains of the small and large intestines. In several cases, the AP limits of Hox gene expression precisely correlate with endoderm organ boundaries (e.g., between the presumptive stomach and the duodenum and between the small and large intestines; see Figure 40.4).

It is clear that the Hox genes have the ability to regulate the AP patterning of the gut. For example, the transgenic misexpression of the *Hox3.1 (Hoxc8)* gene more anteriorly results in profound gastrointestinal tissue malformations and the loss of positional identity (Grapin-Botton, 2005). In the pharyngeal region, the deletion of *Hoxa3* results in the loss of the thymus and of the parathyroid and hypoplasia of the thyroid (Manley and Capecchi, 1995, 1998). Similar gene targeting studies have demonstrated that *Hoxc4* mutants have esophageal defects, that *Hoxa5* is necessary for the development of the stomach, and that *Hoxa13/Hoxd13* compound mutants have hindgut defects (reviewed by Grapin-Botton, 2005). Of these gut-related defects linked to Hox genes, only *Hoxa13* has been shown to specifically function in the endoderm. Novel *Hoxa13* mutations have been associated with hand–foot–genital syndrome, a rare dominantly inherited condition in humans. Consistent with this finding is the fact that the expression of this mutant form of *Hoxa13* in the endoderm of chick embryos results in hindgut and genitourinary patterning defects (de Santa Barbara and Roberts, 2002). Other Hox

mutations that affect gut development *(Hoxa3, Hoxc4,* and *Hoxa5)* are the result of primary defects in gut mesenchyme or neural crest cells that contribute to the developing gut. This highlights the importance of signaling between endoderm and neighboring mesoderm and ectoderm for the proper patterning and development of the gut.

III. SIGNALING PROCESSES INVOLVED IN THE PATTERNING AND FORMATION OF THE FETAL GUT

Despite the cell lineage and molecular evidence suggesting that endoderm cells have positional identity by the end of gastrulation, these cells are not yet committed to specific lineages along the AP axis. In fact, there is increasing evidence that a continuum of signals after the gastrulation stage progressively restrict endoderm cell fate into specific organ lineages. Efforts to understand what regulates these important events have led to the identification of several candidate factors. These include soluble growth factors, such as fibroblast growth factors (FGFs), Wnts, Hedgehogs, and retinoic acid (RA). These signaling pathways regulate the expression of transcription factors, including the ParaHox and Hox genes, which have been discussed previously and which are important mediators of cell fate.

The gastrula stage endoderm is in close proximity with both the mesoderm and the ectoderm, and it has been demonstrated in several species that soluble signals from these adjacent germ layers can influence the AP fate of the endoderm (Horb and Slack, 2001; Le Douarin, 1968; 1988; Wells and Melton, 2000). Experiments in the mouse and the frog demonstrated that endoderm cocultured with different AP populations of mesoderm will adopt the AP character of the cocultured mesoderm (Figure 40.5; Horb and Slack, 2001; Wells and Melton, 2000). Consistent with this is the fact that grafting posterior mouse endoderm, which would normally become hindgut, into the presumptive pancreatic domain of a late gastrula chick embryo causes that endoderm to be respecified to express *Pdx1* (J.M. Wells, unpublished data). These data suggest that endoderm cells at the gastrula stage have not acquired an AP identity but rather that they retain a high degree of plasticity.

A. Signals That Pattern the Gastrula Stage Endoderm

The temporal and spatial expression pattern of several peptide growth factors is consistent with a role in AP patterning. In particular, the primitive streak, which is a structure that defines the posterior of chick and mouse embryos, expresses several FGF and Wnt ligands, including *FGF2, FGF3, FGF4, FGF5, FGF8, Wnt3, Wnt5,* and *Wnt11*. All of these are expressed in the posterior and thus could function in the AP patterning of endoderm. Although the involvement of most of these factors in endoderm patterning has not been established, FGF signaling has been shown to influence the early stages of endoderm patterning as well as to help maintain AP domains in the developing gut tube (Dessimoz et al., 2006; Wells and Melton, 2000).

The FGF family of growth factors is comprised of 23 genes in mammals, and it is known to regulate cellular growth and differentiation processes throughout embryonic development and in adult tissues (Dailey et al., 2005). Of the FGF ligands that are expressed in the late gastrula embryo, only

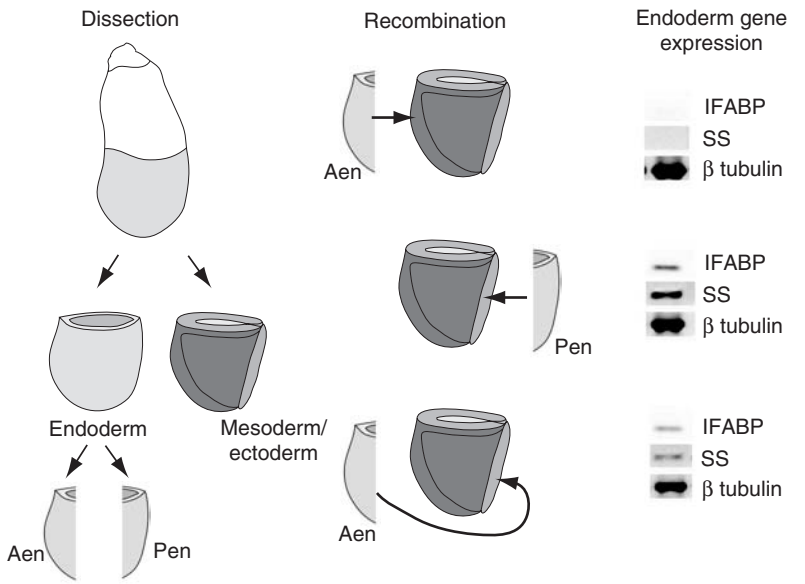

FIGURE 40.5 **Using embryonic explant assays to investigate soluble signals that pattern the endoderm.** Embryonic day 7.5 mouse embryos can be isolated and the germ layers dissected and cultured. The left panel schematically shows the dissection of embryos and the separation of the endoderm, mesoderm, and ectoderm germ layers. These layers can be further dissected into anterior or posterior endoderm. The middle panel illustrates the recombination of anterior endoderm or posterior endoderm with mesoderm/ectoderm. The right panel shows a reverse transcriptase–polymerase chain reaction analysis of several posterior endoderm markers, including intestinal fatty acid binding protein and somatostatin. Anterior endoderm does not normally express posterior markers when it is cultured with anterior mesoderm/ectoderm. However, when it is cultured with posterior mesoderm/ectoderm, it adopts a posterior fate as indicated by the *de novo* expression of intestinal fatty acid binding protein and somatostatin. Posterior endoderm will also adopt an anterior fate when it is cultured with anterior mesoderm/ectoderm (not shown). (Data adapted from Wells and Melton, 2000.)

FGF4 (eFGF in frog) has been implicated in endoderm patterning (Dessimoz et al., 2006; Wells and Melton, 2000). *FGF4* is expressed in the posterior mesoderm and ectoderm adjacent to the presumptive midgut and hindgut endoderm. At this stage, receptors for *FGF4* and the FGF target genes *Sprouty1* and *Sprouty2* are expressed in the endoderm, which suggests that the endoderm is receiving an FGF signal (Dessimoz et al., 2006). In the mouse, embryos lacking either the *FGF4* or the *FGF receptor 1 (FGFR1)* gene have a similar phenotype and arrest at gastrulation (Ciruna et al., 1997; Yamaguchi et al., 1994). These findings suggest that *FGF4* acts via *FGFR1* during gastrulation and that *FGF4* has unique activity that is not compensated for by other FGF ligands.

As a result of early embryonic lethality, the roles of *FGF4* and *FGFR1* in endoderm patterning have not been identified using mouse genetics. However, using mouse endoderm explants and chick embryology, several studies have demonstrated the involvement of FGF4-mediated signaling in endoderm patterning (Dessimoz et al., 2006; Wells and Melton, 2000). In culture and in embryos, *FGF4* protein (but not other FGF ligands) represses anterior cell fate and promotes posterior midgut and hindgut cell fates. The *FGF4*-mediated inhibition of anterior cell identity corresponds with disrupted foregut

morphogenesis, which suggests that a direct link exists between patterning and morphogenesis. Inhibiting FGF signaling *in vivo* results in the loss of *Pdx1* and *Cdx* expression and in the posterior expansion of *Hex1* expression. This suggests that FGF signaling is necessary for promoting posterior cell fate and inhibiting anterior cell fate (see Figure 40.6). Consistent with this is the fact that a *Cdx* homolog in Xenopus, *xCad3,* is a posterior determinant and a direct FGF target (Haremaki et al., 2003). It is also known that different levels of FGF signaling can promote different cell fates along the AP axis (i.e., high doses of *FGF4* protein induce hindgut gene expression, whereas lower doses induce midgut gene expression; Wells and Melton, 2000). Taken together, these studies suggest that FGF signaling represses foregut and promotes midgut and hindgut cell fate in a dose-dependent manner. *FGF8* regulates posterior expression of *FGF4* in the gastrula embryo and *FGF4* was shown to be a direct target of the Wnt signaling pathway in the developing tooth (Kratochwil et al., 2002). Several Wnt ligands are expressed in the primitive streak, suggesting that Wnt signaling could regulate *FGF* ligand expression to promote posterior fate directly via transcription factors such as *Cdx* (see Figure 40.6).

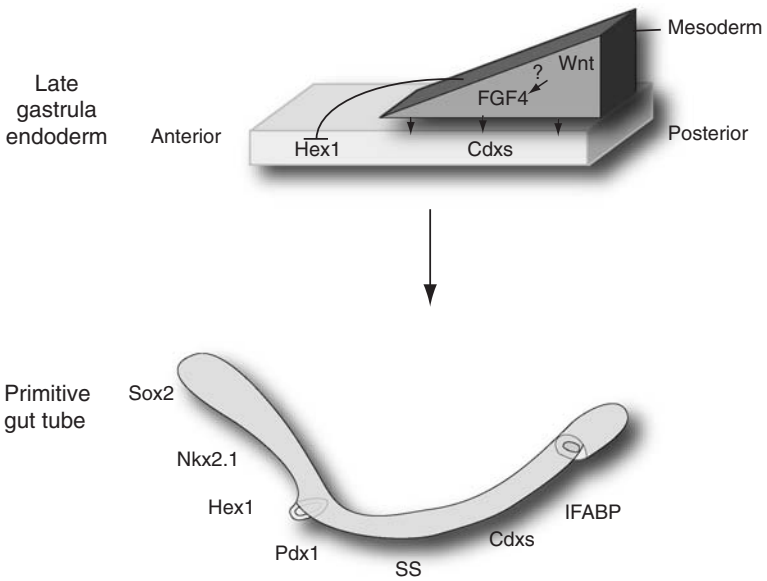

FIGURE 40.6 Signals from the mesoderm at the gastrula stage are necessary for establishing anterior–posterior patterning during early gut tube development. The upper panel schematically shows the endoderm *(light gray)* and the adjacent mesoderm *(drak gray)*. Several fibroblast growth factor (FGF) and Wnt ligands are expressed in the posterior (primitive streak) in the late-gastrula embryo. *FGF4* has been shown to promote posterior gene expression in a dose-dependent manner, with the highest levels promoting the most posterior fates. *FGF4* also represses anterior gene (*Hex1*) expression (at the gastrula stages only). Although it is not shown in the context of endoderm patterning, it is known in other contexts that *FGF4* is a direct target of the canonical Wnt signaling pathway (Kratochwil et al., 2002). The lower panel shows several of the genes that are expressed along the anterior–posterior axis of the primitive gut tube. Many of these genes, including *Nkx2.1, Hex1, Pdx1,* and *Cdx,* are expressed in restricted domains that predict where organ primordia will form. Alterations in *FGF4*-mediated signaling disrupt early endoderm patterning, and they cause morphologic and gene expression changes in the developing foregut, midgut, and hindgut domains (Dessimoz et al., 2006).

B. Establishing Presumptive Organ Domains in the Primitive Gut Tube

1. Foregut Patterning

The foregut endoderm contributes to several organs, including the thyroid, the lungs, the liver, and the pancreas. The dynamic nature of foregut morphogenesis brings the endoderm into proximity with several mesodermally derived tissues that are now known to pattern the foregut. During the early stages of foregut morphogenesis (embryonic day 8 in the mouse; HH stage 6 in the chick), the ventral foregut endoderm is adjacent to the cardiac mesoderm. During the later stages (embryonic day 8.5 in the mouse; HH stage 9 in the chick), the endoderm comes in contact with the septum transversum mesenchyme and the endothelial cells of the ventral veins. The dorsal foregut endoderm is brought into contact with the notochord and the dorsal aorta. Using a combination of tissue recombination, embryology, and genetics, specific signaling molecules emanating from these tissues have been shown to pattern the foregut.

The liver derives exclusively from the ventral foregut, and it is the first foregut derivative to form. Studies in the chick and the mouse suggest that the initial specification of the liver depends on signals from adjacent cardiac mesoderm (reviewed by Grapin-Botton, 2005). In mouse ventral foregut explant cultures, it was shown that the cardiac mesoderm signals to the ventral foregut to induce liver and that this occurs between the three- and eight-somite stages. Interestingly, in more posterior regions of the gut, negative signals were necessary to prevent precocious liver albumin gene expression in nonhepatic trunk endoderm. When trunk endoderm was removed from these negative signals, it expressed the liver marker albumin. Although the direct targets of the cardiac signal have not been identified, there are several transcription factors that are expressed at high levels in prehepatic ventral endoderm, including *Hex1, Gata* factors, *Foxa1,* and *Foxa2,* all of which are known to play roles in liver development (McLin and Zorn, 2006).

Foregut explant cultures have been used to identify specific signaling molecules produced by cardiac mesoderm, particularly *FGF1 (aFGF)* and *FGF2 (bFGF)* proteins, which pattern the ventral foregut into liver, pancreas, and lung domains (Deutsch et al., 2001; Gualdi et al., 1996; Serls et al., 2005). From these studies, a model has been proposed whereby different concentration thresholds of FGF ligands pattern the ventral foregut into these different lineages, with high levels promoting liver, moderate levels promoting lung, and low levels promoting ventral pancreas. It is also possible that the length of time that an endoderm cell is exposed to the cardiac mesoderm influences its fate. For example, cell lineage experiments show that ventral foregut endoderm cells adjacent to cardiac mesoderm end up distributed along the AP length of the foregut at later stages (Tremblay and Zaret, 2005). Therefore, it is possible that endoderm cells that remain in proximity to cardiac mesoderm longer adopt a liver fate, that cells that migrate posteriorly away from the cardiac mesoderm become ventral pancreas, and that cells that migrate anteriorly become lung (Deutsch et al., 2001; Serls et al., 2005).

RA is a key regulator of embryonic patterning and differentiation. Several reports in frogs, fish, chicks, and mice have implicated RA signaling in the development of foregut derivatives including in the lung, the liver, and the pancreas. RA signaling is shown to be important for the global AP patterning of the foregut endoderm and thus that it affects the development of

multiple organs. For example, it has been shown that RA treatment is sufficient to cause an anterior shift of *Pdx1* expression in embryonic chick explants of lateral endoderm plus mesoderm (Kumar et al., 2003). However, in this example, the presence of mesenchyme was required, which suggests that the effect on endoderm was indirect. Other growth factors had similar posteriorizing activity, including bone morphogenetic proteins (BMPs) and activin. Thus, it is possible that RA regulates the mesenchymal expression of these factors, which then signal to endoderm. A global foregut patterning role of RA was also suggested in the mouse, because embryos deficient in retinaldehyde dehydrogenase 2 *(Raldh2)* and lacking active RA signaling in the foregut region fail to develop the lungs, the stomach, and the dorsal pancreas, and they have impaired liver growth (Wang et al., 2006). RA signaling in the foregut was shown to act upstream of *Pdx1, FGF10,* and Hox genes, which suggests that it is globally affecting the early patterning of the foregut.

There are reports in zebrafish, Xenopus, and the mouse suggesting that RA signaling plays patterning-independent roles in early pancreas development. Studies in Xenopus suggest that activation of the RA pathway does not effect the AP expression of *Pdx1 (Xlhbox8)* as it does in the chick (Zeynali and Dixon, 1998); however, it may affect later aspects of pancreas development (Chen et al., 2004). The pancreas develops from the dorsal and ventral foregut endoderm, and, interestingly, mouse embryos deficient for the RA-synthesizing enzyme *Raldh2* fail to initiate dorsal pancreas development, but ventral pancreas development initiates normally (Martin et al., 2005). The development of the pancreatic mesenchyme is also deficient in these animals, which suggests that pancreatic defects may be the result of a loss of a mesenchymal signal. In zebrafish, however, the disruption of RA receptor function, specifically in the endoderm, perturbs normal pancreas development; this suggests a cell-autonomous role for RA signaling in the endoderm (Stafford et al., 2006). The apparent discrepancies involving the role of RA signaling in the development of various foregut derivatives may be the result of species differences, or they could suggest that RA signaling has multiple roles during the early stages of foregut patterning and the early stages of organ development.

The role of Hedgehog signaling in pancreas development is more extensively discussed elsewhere in this book (see Chapter 42). However, there is evidence to suggest that a primary role of Hedgehog signaling is patterning the gut tube. During the early stages of gut tube development (embryonic days 8.5–9 in the mouse), Sonic hedgehog *(Shh)* is broadly expressed by the endoderm along the AP axis. As the thyroid, thymus, parathyroid, and pancreas organ rudiments begin to form, *Shh* expression is repressed in these budding organs. In the case of the pancreatic bud, the repression of *Shh* depends on signals from the notochord, which include *FGF2* and activin (reviewed by Kim and Hebrok, 2001). Surprisingly, in transgenic mice in which *Shh* was misexpressed in the *Pdx1* domain, the initiation of pancreas development and the formation of the endocrine and exocrine lineages were relatively normal. However, the adjacent mesenchyme underwent a homeotic transformation toward an intestinal fate, forming intestinal smooth muscle and interstitial cells of Cajal. Gene targeting studies in mice also show that *Shh* mutants display intestinal transformations of the stomach epithelium as evidenced by the expression of duodenal markers in the stomach (Ramalho-Santos et al., 2000).

Studies in the chick have also suggested that *Shh* is involved in the patterning of the foregut and in the establishment of the gizzard/stomach organ boundaries. This study suggests that *Shh* from the endoderm regulates the expression of *Nkx2.5* in the gizzard and that ectopic *Shh* expression causes inappropriate *Nkx2.5* expression in the pancreas. *Shh* did not directly induce *Nkx2.5;* rather, it induced the expression of *BMP4* in adjacent mesenchyme. The cell-autonomous activation of BMP signaling in the mesenchyme through the expression of activated BMP receptors was sufficient to cause the upregulation of *Nkx2.5* in the gizzard, and it resulted in perturbed smooth muscle differentiation.

C. Patterning the Anterior Foregut/Pharyngeal Domain

The anterior portion of the foregut is commonly referred to as the *branchial* or *pharyngeal region*. This region of the vertebrate embryo is anatomically hallmarked by a series of bilateral bulges called *arches,* which decrease in size from anterior to posterior (Figure 40.7). The pharyngeal endoderm lines the interior of the pharyngeal arches, and it will transiently fuse with the surface ectoderm to form pouches (interior endoderm) and clefts (exterior ectoderm) that mark the anterior and posterior boundaries of the arches. Pharyngeal pouches form from anterior to posterior, and they exhibit regionally restricted identity as marked by the differential expression of *FGF8* and *paired box 1 (Pax1)*. During normal development, the pharyngeal endoderm will contribute to the trachea, the esophagus, the thymus, the parathyroid, the thyroid, the ultimobranchial bodies (which form the calcitonin-producing parafollicular cells of the thyroid), and the taste buds. However, multiple human disorders are associated with abnormal pharyngeal development, including esophageal atresia, tracheoesophageal fistula, VACTERL, and the DiGeorge and velocardiofacial syndromes, and this emphasizes the clinical importance of understanding anterior foregut patterning. Furthermore, from a teratologic standpoint, pharyngeal region morphology appears to be particularly sensitive to alcohol exposure, possibly as a result of the competitive interactions of alcohol with RA-metabolizing enzymes present in pharyngeal tissues (Wang, 2005).

One key role of pharyngeal endoderm is as a regional source of patterning signals for the adjacent mesoderm and ectoderm (reviewed by Graham and Smith, 2001). Signaling molecules expressed by endoderm include *Shh,* multiple members of the FGF family, BMPs, the BMP antagonists *chordin* and *noggin,* and several RA receptors. Although it is not known exactly how these pathways intersect to pattern the pharyngeal region, we will later discuss a potential patterning model that is based on the analysis of mouse mutants and gene expression studies (see Figure 40.7).

Both *FGF3* and *FGF8* are expressed in the pharyngeal pouch endoderm, and both are required for proper pouch formation (Crump et al., 2004). Mutants lacking *FGF8* and *FGFR1* have severe phenotypes that exhibit deletions of both the third and fourth arches and that result in the loss of the thymus and the parathyroid (Abu-Issa et al., 2002; Trokovic et al., 2003). *FGF8* expression in the pharyngeal endoderm is lost in embryos that lack *T-box 1* (*Tbx1*; see Chapter 16). Which suggests that *Tbx1* is upstream of *FGF8*. Furthermore, *Tbx1* mutations have been shown to contribute to

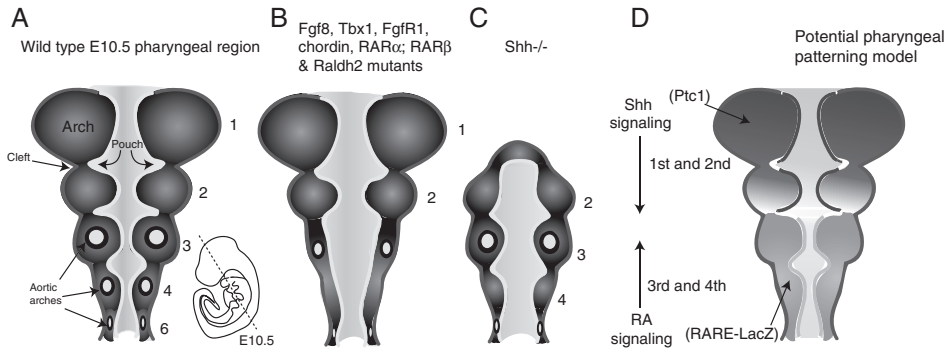

FIGURE 40.7 **Patterning the anterior foregut/pharyngeal domain. A,** Ventral/coronal view of normal pharyngeal arches. The outline of an embryonic day 10.5 embryo shows the corresponding plane of section *(dotted line)* of the larger image. The endoderm is in yellow, the surface ectoderm is blue, and the mesenchyme of the arches is red. Arches are numbered 1 through 4 and then 6, from anterior to posterior. The pouches and clefts are numbered by that of the preceding arch. **B,** The embryonic day 10.5 pharyngeal phenotype of the *FGF8, Tbx1, Fgfr1, chordin, RARα/RARβ,* and *RALDH2* mutant mice in which the caudal third and fourth arches are missing. **C,** The embryonic day 10.5 pharyngeal phenotype of the *Shh* null mutant mice in which the first arch is missing and the second is severely hypomorphic. (Note: The *Shh* mutant embryos initiate the development of the first and second arches on embryonic day 9.5, but they lose the first arch by embryonic day 10.5, and the second arch continues to atrophy.) **D,** A putative model for the anterior–posterior patterning of the pharyngeal region, with *Shh*- and RA-mediated signals regulating the anterior and posterior regions of the pharyngeal region.

DiGeorge syndrome in humans (Jerome and Papaioannou, 2001). Patients with DiGeorge syndrome often have numerous defects in craniofacial and cardiovascular development and defects in organs that are derived from the pharyngeal pouch endoderm, including the thymus and the parathyroid.

Considering the importance of *Tbx1* in human disease, there is much interest in the signaling pathways that regulate *Tbx1* expression, and several reports indicate that *Tbx1* may be regulated by the morphogen *Shh* (Riccomagno et al., 2002; Yamagishi et al., 2003). *Tbx1* expression in the pharyngeal region is lower in $Shh^{-/-}$ mutant mouse embryos, a *Tbx1*-promoter–driven LacZ transgene showed reduced expression in an $Shh^{-/-}$ background, and exposing pharyngeal mesenchyme to exogenous *Shh* protein resulted in the ectopic expression of *Tbx1*. However, several phenotypic differences between *Tbx1* and *Shh* mutants suggest that the Hedgehog pathway is more critical for the development of the anterior pharyngeal region, whereas the *Tbx1* phenotypes are predominantly in the caudal pharyngeal arches. For example, *Shh* mutants form a thymus (unlike the *Tbx1* null embryos), whereas parathyroid development is perturbed in both mutants (Ivins et al., 2005; Moore-Scott and Manley, 2005). This phenotypic overlap suggests that *Shh* may act as a modifier of *Tbx1* expression in the pharyngeal region.

In addition to its role in global endoderm patterning, the RA pathway, which is mediated by the retinoid receptors (RARs) RARα, RARβ, and RARγ, is essential for the patterning of the pharyngeal endoderm. RA has been shown to regulate *Shh* and *Tbx1* expression as well as the expression of several Hox genes, all of which are essential to the AP patterning of the pharyngeal endoderm (reviewed by Graham and Smith, 2001). In the case of *Hoxb1* gene, an RA-inducible enhancer has been identified ($RAIDR_5$; Graham and Smith, 2001; Grapin-Botton, 2005). This element drives broad

expression of *Hoxb1* throughout the foregut on embryonic day 8.5, but it becomes restricted to the pharyngeal endoderm of the anterior foregut on embryonic day 9.5. In addition, exposing embryos to exogenous RA causes an anterior expansion of *Hoxb1*, suggesting that RA signaling regulates foregut pattern by directly regulating the transcription of *Hoxb1*. In *Tbx1* mutants, *Raldh2* expression expands anteriorly, which suggests that RA signaling is regulated by *Tbx1* (Ivins et al., 2005). Embryos exposed to a pan-RAR antagonist, a pan-RAR agonist, vitamin A, or RA lose the caudal third and fourth pharyngeal arches. These treatments phenocopy those observed in the compound *RARα* and *RARβ* knockouts and the hypomorphic *Raldh2* mutant, and they illustrate the importance of proper RA signaling in pharyngeal region patterning (Niederreither et al., 2003; Vermot et al., 2003).

BMPs are members of the transforming growth factor β superfamily of growth factors, and they have also been implicated in patterning and pharyngeal organogenesis. *BMP2, BMP4,* and *BMP7* as well as the BMP antagonists *noggin* and *chordin* are expressed in the pharyngeal region in all three cell types, including the endoderm. The *chordin* null, like the *FGF8* and *Tbx1* null and the compound *RARα/RARβ* mutants, fails to form the posterior arches, which implies that the inhibition of BMP signaling is part of a complex pathway that is necessary for the posterior patterning of the pharyngeal endoderm (Bachiller et al., 2003).

These data establish that *Tbx1, FGF8,* RA, and *chordin* are all required for the formation of the posterior pharyngeal region, whereas the anterior mutant phenotype in the *Shh* mutants suggest an oppositional role for Hedgehog signaling in the AP patterning of the pharyngeal endoderm (see Figure 40.7, B and C; Graham and Smith, 2001). Moreover, *Patched1 (Ptc1)* is an *Shh* transcriptional target that is expressed in the first and second arch, whereas an RA-responsive-element–driven LacZ transgene is expressed in the third and fourth arches (see Figure 40.7, *D*), which supports the idea of Hedgehog signaling in the anterior arches and RA signaling in the posterior arches. It is interesting that none of the specific components of the Hedgehog, FGF, or RA signaling pathways are expressed in restricted pharyngeal domains along the AP axis. In fact, many are expressed in discrete domains within the endoderm or throughout the entirety of the pharyngeal endoderm, mesoderm, and ectoderm. It is not known how the activation of the Hedgehog and RA pathways is restricted to specific AP arches. Clearly, a complex interaction of the pathways occurs that has yet to be fully defined and thus requires further study.

D. Midgut and Hindgut Patterning

Early during the development of the gut tube, the posterior endoderm folds ventrally generating the hindgut. The midgut and hindgut form the small and large intestines and the hindgut also contributes to the cloaca, which forms the urogenital tract. Studies have begun to identify the molecular mechanisms that regulate posterior identity in the hindgut. As discussed previously, FGF signaling before gut tube morphogenesis is involved in the establishment of posterior endoderm identity (Dessimoz et al., 2006). These studies also found that FGF signaling is required to maintain the AP expression boundaries of *Pdx1* and *Cdx* genes at later stages of gut tube development. Shifting *FGF4*-mediated signaling to the anterior caused an anterior shift in

the expression of *Pdx1* and *Cdx* genes in the primitive gut tube, which suggests that anterior regions of the gut tube were transformed to a posterior fate. Conversely, inhibiting FGF signaling caused a loss of posterior identity. Moreover, these studies demonstrated that FGF signaling was acting directly on the endoderm. Several other signaling molecules, including BMP, activin, and RA, also have the ability to posteriorize the lateral endoderm that contributes to the ventral gut, but only in the presence of lateral plate mesoderm; this suggests that these factors may act indirectly (Kumar et al., 2003).

These findings illustrate the fact that gut patterning involves reciprocal signaling between endodermally derived epithelium and mesodermally derived tissues, including the notochord and the gut mesenchyme. Additional reciprocal interactions have been identified between the hindgut endoderm and the mesoderm that result in spatially restricted Hox gene expression in the hindgut and the establishment of posterior identity in the endoderm (reviewed by Grapin-Botton, 2005; Wells and Melton, 1999). Specifically, hindgut endoderm expresses *Shh,* which is sufficient to induce *BMP4* and *Hoxd13* expression in adjacent posterior mesoderm but not in the more anterior mesoderm adjacent to the stomach. If *Hoxd13* is misexpressed in the more anterior mesoderm, the adjacent stomach endoderm is transformed into an intestinal type of endoderm as assayed by morphology and marker expression. The molecules that transmit the signal from *Hoxd13*-expressing mesenchyme to endoderm are unidentified.

The role of canonical Wnt signaling in regulating the homeostasis of intestinal epithelium is well established. Recently, several reports have implicated this pathway in the embryonic development and patterning of the gastrointestinal tract. Numerous Wnt signaling components are expressed along the AP axis of the gastrointestinal tract throughout its development (Theodosiou and Tabin, 2003). Functional evidence implicating Wnt signaling in gut development has come from studies of *T cell factor/Lymphoid enhancer factor 1* (TCF/LEF) family of transcription factors, which are downstream effectors of the canonical Wnt pathway. In the chick, the expression of dominant-negative *TCF4* in the mesenchyme caused secondary defects in the differentiation of gizzard epithelium. In the mouse, *TCF1/TCF4*$^{-/-}$ embryos have defects in hindgut expansion and an anterior transformation of the duodenum (Gregorieff et al., 2004). Although these studies implicate canonical Wnt signaling in gut tube patterning, it is not known whether TCF factors act in the endoderm, the mesoderm, or both.

IV. IMPACT OF GENOMICS ON OUR UNDERSTANDING OF EARLY ENDODERM ORGANOGENESIS

Current technology and database organization make it possible to generate a quantitative catalog of all of the genes expressed at each step of organ development, from the specification of embryonic endoderm to the formation of functioning adult cell types such as insulin-producing β cells (see also the chapter by Gannon in this book). These databases allow researchers to analyze the cell-type–specific expression of a particular gene or to look for entire functional classes of genes, such as DNA-binding factors expressed in the developing or adult cell types. More importantly, these types of analyses

should provide a molecular foundation for future studies of organ development and information about how to direct the differentiation of stem cells into therapeutically important cell types, such as pancreatic β cells. Recently, Affymetrix microarrays were used to perform a quantitative gene-expression analysis of highly purified cells isolated from four key stages of endocrine pancreas development (Gu et al., 2004). The stages and cells analyzed in this experiment were as follows: (1) embryonic day 7.5 prepancreatic endoderm; (2) embryonic day 10.5 pancreatic progenitor cells as defined by *Pdx1* expression; (3) embryonic day 13.5 endocrine progenitor cells as defined by *Ngn3* expression; and (4) adult islets of Langerhans. This approach was highly successful for generating a gene expression database of the developing endocrine pancreas. More importantly, this study identified many novel genes expressed at each stage of endocrine pancreas development, and the functional analysis of one of these genes demonstrated its involvement in endocrine cell development.

For the purpose of this chapter, we will focus on the stages of gut development during which the patterning of endoderm results in the establishment of the *Pdx1* domain. The expression of more than 12,000 genes was measured simultaneously to identify the genes induced as endoderm is specified toward the pancreatic lineage. Genes that showed a greater than three-fold expression change during this endoderm patterning stage were grouped and analyzed. For simplicity, we will focus on two classes of regulatory molecules that control cell fate: transcription factors and growth factors/receptors. FGF, *notch,* and activin signaling pathways have been implicated in various aspects of early pancreas specification (reviewed by Kim and Hebrok, 2001), and DNA microarray expression data are consistent with these findings showing that *Pdx1* cells express *FGFR1, FGFR2,* and *FGFR4* as well as *activin receptor IIb* (data not shown), *notch 3,* and *delta-like 1*. These studies also identified a number of components of the Wnt signaling pathway that were expressed in the early pancreas, including Wnt receptors, *frizzled2, frizzled4,* and secreted forms of *frizzled* that antagonize Wnt signaling. The identification of Wnt signaling components in the early pancreas prompted several groups to perform functional studies of this pathway that have demonstrated its importance in pancreas development (Dessimoz et al., 2005; Murtaugh et al., 2005; Wells et al., 2007).

These signals culminate in the expression of several transcription factors in the pancreatic endoderm, including *Pdx1, Hlxb1,* and *Ptf1a/p48,* all of which are necessary for proper pancreas development (reviewed by Grapin-Botton, 2005). Our analysis detected these genes in early pancreatic cells (data not shown), and we found numerous other transcription factors, most of which were not previously described in the developing pancreas. These transcription factors include the basic helix–loop–helix type *(Id, Foxa2,* and *Foxa3)* and the homeobox type *(Barx1* and *Hoxa5)* as well as other classes. The function of most of these factors in pancreas development has not been determined.

V. CLINICAL APPLICATIONS FOR STUDIES IN ENDODERM ORGANOGENESIS

A significant number of diseases affect endodermally derived organs, particularly the lungs, the liver, and the pancreas. Moreover, childhood diseases such as asthma and diabetes, which affect the respiratory and gastrointestinal tracts, are increasing at an alarming rate. The study of the molecular mechanisms

underlying endoderm organogenesis is a relatively new field. Nonetheless, experiments in model organisms have already led to the identification of genes involved in diseases of the endoderm. The exciting new fields of regenerative medicine and tissue engineering have greatly benefited from research in developmental biology. For example, it has become increasingly apparent that some adult organs reactivate embryonic pathways during the process of regeneration. Injury to the lung and tracheal epithelium causes a regenerative response that is accompanied by a dramatic increase in the expression of embryonic endoderm regulatory genes, including *Sox17, Foxa1, Foxa2,* and others, and some of these genes may aid in regeneration (Park et al., 2006a, 2006b). Another means of translating embryonic studies into clinical applications is through the directed differentiation of embryonic stem cells into endoderm derivatives. It has recently been shown that the embryonic growth factor activin can direct the differentiation of human embryonic stem cells into endoderm in culture (D'Amour et al., 2005). This exciting advance will now allow researchers to study how human embryonic stem cell–derived endoderm is patterned and ultimately directed into specific organ lineages. A renewable source of endodermally derived tissues would have a profound impact on therapeutic approaches involving transplantation-based therapies.

SUMMARY

- The endoderm germ layer in the late-gastrula embryo (embryonic day 7.5 in the mouse; HH stages 4 and 5 in the chick) is a single layer of cells that are unspecified. Within 2 days of development (embryonic day 9.5 in the mouse; HH stage 18 in the chick), the endoderm has formed a primitive gut tube with budding organ primordia.
- The late-gastrula endoderm is regionalized into axial and lateral domains along the AP axis that give rise to the foregut, the midgut, and the hindgut.
- By the early somite stage (embryonic day 8.5 in the mouse; HH stage 10 in the chick), the developing gut tube has become highly patterned at the molecular level. Genes are expressed in overlapping and distinct domains that predict where organ buds will form.
- Several signaling pathways are involved in these early patterning mechanisms, including the FGF, Hedgehog, BMP, RA, and Wnt pathways.
- The disruption of these early patterning mechanisms can directly lead to defects in the development of endoderm organs, including the thymus, the parathyroid, the lungs, the liver, the pancreas, and the intestines.
- Genomics strategies are being used to identify new molecular pathways involved in endoderm organogenesis.
- Information from endoderm organ development is being used to differentiate human embryonic stem cells into endoderm organ cell types that ultimately could be used to treat degenerative diseases such as diabetes.

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GLOSSARY

Endoderm
The inner layer of cells in embryonic development that gives rise to digestive and respiratory organs. Note that, early in the development of many vertebrate embryos, the endoderm is initially the outermost layer of cells.

Foregut
The anteriormost of the three divisions of the digestive tract (the foregut, the midgut, and the hindgut).

Hindgut
The posteriormost region of the digestive tract.

Liver
The largest gland in the human body, with roles in digestion, glucose regulation and storage, blood clotting, and the removal of wastes from the blood.

Lung
An organ that contains sac-like structures in which blood and air exchange oxygen and carbon dioxide.

Organogenesis
The development of organs during embryonic development.

Pancreas
A gland in the abdominal cavity with both exocrine and endocrine function that secretes digestive enzymes into the duodenum and that also secretes the hormones insulin and glucagon into the blood.

Parathyroid
A calcium homeostatic organ that secretes parathyroid hormone.

Patterning
The act of subdividing embryos or tissues into distinct domains along the embryonic axes.

Primordia
Cells, tissues, or organs at the earliest stage of development.

Thyroid
An endocrine organ with the functions of regulating metabolism, growth, and development.

Ultimobranchial bodies
Small glands that develop separately from the thyroid that will fuse with the thyroid and form the parafollicular cells of the thyroid.

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RECOMMENDED RESOURCES

Developmental Biology Online:
http://www.uoguelph.ca/zoology/devobio/dbindex.htm
Chicken Developmental Stages:
http://embryology.med.unsw.edu.au/OtherEmb/chick1.htm
Embryo Images:
http://www.med.unc.edu/embryo_images/
Jackson Laboratory Gene Expression Database:
http://www.informatics.jax.org/menus/expression_menu.shtml
International Society for Stem Cell Research:
http://www.isscr.org
The Wnt Homepage:
http://www.stanford.edu/%7Ernusse/wntwindow.html

41

DEVELOPMENTAL GENETICS OF THE PULMONARY SYSTEM

THOMAS J. MARIANI

Department of Medicine, Brigham and Women's Hospital and Pulmonary Bioinformatics, The Lung Biology Center, Harvard Medical School, Boston, MA

INTRODUCTION

The mammalian lung develops as a lateral bud from the ventral foregut endoderm between the developing liver and the thymus. Lung development initiates at 5 weeks of gestation in the human and at 9 days of gestation in the mouse, and it is understood as proceeding through four discrete, subsequent stages: pseudoglandular, canalicular, saccular, and alveolar (Figure 41.1). Each of these stages is defined morphologically, and each encompasses distinct structural, cellular, and regulatory features. During the first 30 weeks after initiation in the human, the bud grows into a branched tubular structure that is reminiscent of other glandular organs to comprise the conducting airways. Until 36 weeks of gestation in the human or postnatal day 4 in the mouse, these tubes end in sacs that are incapable of efficient gas exchange. Birth occurring before or at this time (if untreated) is associated with increased morbidity and mortality as a result of lung immaturity. During the last few weeks of gestation and the first few years of life, these primitive sacs undergo a morphologic process that results in the development of mature alveoli. This process involves a dramatic increase in the surface area of the lung as a result of the formation and elongation of buds or secondary crests off of the walls of the primary sacs. Numerous adult lung diseases involve the destruction of the alveolar space. Therefore, a thorough understanding of the regulation of lung development, including alveolar formation and maintenance, could identify means to promote the maturation, limit the destruction, and support the regeneration of lung function.

In the rodent, lung development initiates at mid-gestation and proceeds in a delayed fashion as compared with that seen in humans throughout gestation. In fact, a considerable amount of lung maturation, including the entire process of alveogenesis, occurs postnatally in the rodent. Parallels between

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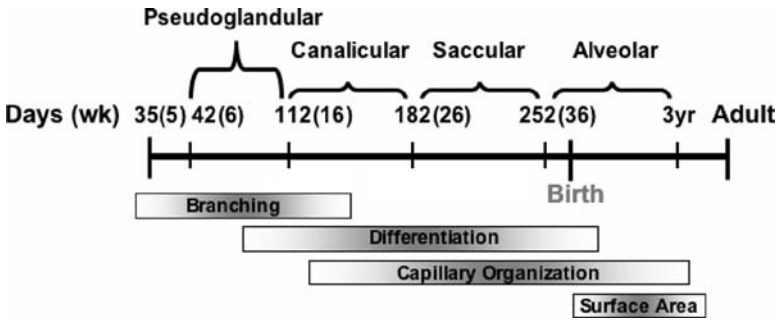

FIGURE 41.1 A timeline for human lung development. Mammalian lung development occurs in discrete morphologic stages. The name and timing of the individual stages are shown, along with a general description of the cellular and molecular processes that occur during the different stages.

the distinct morphologic events and regulatory mechanisms that occur during mammalian lung development have been appropriately drawn with vertebrate limb development and *Drosophila melanogaster* larval tracheal development. The regulation of these morphogenic processes has been more fully defined (Johnson et al., 1994; Tickle, 1999; Capdevila and Izpisua Belmonte, 2001; Ghabrial et al., 2003; Cabernard et al., 2004), and this has served as a basis for the identification of many of the mechanisms that contribute to mammalian lung development.

This chapter will focus on the principal regulatory mechanisms governing the initiation of lung formation, airway branching morphogenesis, sacculation, respiratory epithelial differentiation, and alveolar formation. Data derived from mouse genetics will be reviewed, and information relevant for human disease will be highlighted. Other recent review articles can be referred to for additional information (Warburton et al., 2005; Cardoso and Lu, 2006).

I. LUNG SPECIFICATION AND SYMMETRY

The lung is specified within the developing foregut endoderm by a process that is incompletely defined, but it requires HNF3β, Gli, Shh, and retinoic acid (RA) signaling (Ang and Rossant, 1994; Motoyama et al., 1998; Litingtung et al., 1998; Mendelsohn et al., 1994; Desai et al., 2004). The early commitment of distinct tracheal and respiratory lineages occurs. This is exemplified by tracheal formation, but lung agenesis in cases of deficiencies of either fibroblast growth factor (FGF)-10 or its cognate receptor FGF receptor (FGFR)-2 (Peters et al., 1994; Min et al., 1998; Sekine et al., 1999; De Moerlooze et al., 2000). Although HNF3β specifies completion of the foregut, these other molecules that regulate lung bud initiation appear to comprise a regulatory module, with Gli acting as a regulator of the Shh pathway and RA promoting FGF signaling. Although tracheal malformations are not uncommon in humans, the genetic bases for these defects are presently poorly defined (see Chapter 40).

Mammalian lungs are obviously asymmetric, differing in the number of lobes in the left (three in humans, one in mice) and right lungs (two in humans, four in mice). The specification of lung symmetry occurs through the same mechanisms used to define the left–right axis of the whole organism;

principally the transforming growth factor-β (TGF-β)–related molecules Nodal, ACVR2, Lefty-1, and Lefty-2, along with the homeobox gene Pitx2. Lefty-1 appears to initiate the promotion of "left sidedness," whereas Pitx2 promotes lobar simplification (Kitamura et al., 1999). Recent data strongly suggest that Fog2/GATA signaling is necessary for proper lobar septation (Ackerman et al., 2005); the same study also implicates Fog2 as a potential genetic determinant of lung hypoplasia and congenital diaphragmatic hernia in humans (see Chapter 37).

II. BRANCHING MORPHOGENESIS

During the past decade, genetic dissection has provided tremendous insight into the regulation of airway branching morphogenesis. Numerous regulatory pathways contributing to this process, including epidermal growth factor (EGF), TGF-β/bone morphogenetic protein (BMP), and FGF signaling have been identified. Although EGF receptor mutants display severely diminished branching and blunted lung development (Warburton et al., 1992; Miettinen et al., 1997), this is likely the result of reduced cellular proliferation (Goldin and Opperman, 1980).

BMP-4 and FGF-10 play essential roles in the coordination of branching morphogenesis by specifying the timing and location of bud/tubule growth and elongation or arrest and branching. Sutherland et al. (1996) were among the first to appreciate the critical role played by FGF signaling during branching morphogenesis. Using larval tracheal development in *Drosophila melanogaster* as a model system, they identified dynamic waves of expression for both the Drosophila FGF ligand breathless and its receptor, branchless (see Chapter 21). The activation of this system is essential to drive tubule elongation and branch integration. Detailed studies in mice have shown evolutionary conservation of this regulatory process, with FGF-10 and FGFR-2 serving as the relevant ligand and receptor, respectively (Bellusci et al., 1997). In fact, FGF-10 can promote the direction of elongation of epithelial tubules in rodent lung explant cultures (Park et al., 1998).

The fine-tuning of FGF signaling is provided by Sprouty-2 and -4 as well as the Shh pathway. The Sproutys act to limit the effects of FGF signaling by inhibiting receptor tyrosine kinase activity (Mason et al., 2006), thereby limiting bud growth and/or restricting sites of bud branching. Their potential to contribute specifically to lung branching morphogenesis was initially recognized during studies of Drosophila (Hacohen et al., 1998). In mammals, the inhibition of Sprouty activity leads to increased branching *in vitro* (Tefft et al., 1999), whereas overexpression results in decreased branching *in vivo* (Mailleux et al., 2001; Perl et al., 2003).

Shh signals through patched/smoothened to regulate Gli activity. Like Sprouty, Shh acts to limit bud elongation by repressing FGF signaling at the bud tip. In the absence of Shh, the pattern of branching is severely disrupted (Pepicelli et al., 1998). Likewise, ectopic overexpression of Shh results in abnormal lung development (Bellusci et al., 1997). A deficiency in Hip1, which is a protein that interacts with and inhibits the function of Shh, results in increased Shh activity and decreased branching (Chuang et al., 2003).

BMP-4 appears to be involved in the propagation of FGF-10–related branching morphogenesis signaling centers that specify branch initiation and

outgrowth sites. BMP-4 is expressed in the lung bud epithelium at the tip of the growing bud, which is juxtaposed to the FGF-10 expression in the surrounding mesenchyme (Bellusci et al., 1996). BMP-4 appears to signal the inhibition of elongation and to promote branching (Weaver et al., 2000). This may function (at least in part) by promoting the accumulation of extracellular matrix molecules, such as fibronectin, which serves as a physical barrier to form branching clefts (Sakai et al., 2003). Recent data indicate that BMP-4 can additionally or alternatively have a positive effect on the promotion of branching (Bragg et al., 2001).

Wnt signaling is becoming more appreciated as a component part of the regulation of lung development. Numerous Wnts and their receptors are expressed in the developing lung. As will be discussed later, Wnts appear to have a major role in the regulation of pulmonary vascular development. Within the context of branching morphogenesis, the treatment of lung bud cultures with Dkk1 (a Wnt antagonist) has been shown to inhibit branching, apparently by reducing fibronectin-dependent cleft formation (De Langhe et al., 2005).

Although branching morphogenesis is an essential component of the developmental process of the lung, deficiencies in this process are largely irrelevant to human disease, because they are completely incompatible with life. The notable exceptions are those processes that contribute to tracheal development, which underlie conditions such as tracheal–esophageal fistula, and those that contribute to lobar septation and diaphragm development, which may serve as genetic determinants for some cases of congenital diaphragmatic hernia and lung hypoplasia.

III. SACCULATION AND EPITHELIAL CELL DIFFERENTIATION

At 26 weeks of human gestation or embryonic day 17.5 in the mouse, the lung undergoes the process of sacculation, which dramatically changes the distal architecture of the lung. A predominant feature of sacculation is the flattening of distal airway epithelial cells; this process is regulated by numerous factors, such as GATA-6, Nkx2.1, HNF3β, C/EBPa, glucocorticoid hormones, and FGFs (Cardoso, 2000). These and other morphologic changes are accompanied by the initial expansion of the eventual dozens of distinct cell types that will occupy the pulmonary system.

A proximal–distal axis for the patterning of epithelial cell fate is established before sacculation, with BMP-4 playing an important regulatory role (Weaver et al., 1999). BMP-4 may participate in the signaling that is necessary to maintain developing lung epithelium in either an undifferentiated or distally (airspace) committed state as defined by surfactant protein C (SPC) expression. Additionally, the inhibition of BMP-4 promotes a proximal airway phenotype as defined by Clara cell secretory protein (CCSP; uteroglobin) expression. Wnt/β-catenin signaling also appears to be crucial to establishing this discrete proximal versus distal epithelial fate (Mucenski et al., 2003; Shu et al., 2005). In both cases, the disruption of the signaling leads to the "proximalization" of the distal lung epithelium, thereby suggesting that these pathways function to maintain cells in a distal phenotype. Conversely, Nkx2.1, which is the earliest marker of lung specification, appears to be necessary for the establishment of either proximal or distal fate. Deficiency in Nkx2.1 leads to severe abnormalities in lung development, with the complete absence

of both SPC and CCSP. Many other molecules and pathways contribute to the specification of individual cell fates, of which we currently know very little. Two examples are the identification of the necessity of Foxj1 for the differentiation of ciliated cells (Chen et al., 1998; Brody et al., 2000) and of Foxa2 for the specification of goblet cells (Wan et al., 2004).

IV. MESENCHYMAL DIFFERENTIATION AND VASCULAR DEVELOPMENT

Lung parenchymal fibroblasts, airway and vascular smooth muscle, pleural mesothelial, endothelial, and vascular support cells (pericytes) are all of mesenchymal origin, arising primarily from the lateral plate mesoderm. A thorough understanding of the regulation of the expansion and specification of cells of mesenchymal origin has lagged behind that available for epithelial cells. However, recent data have identified numerous pathways that contribute to the process of mesenchymal cell proliferation and differentiation. Platelet-derived growth factor (PDGF) signaling is understood to play a dominant role in the promotion of lung mesenchymal cell development, and PDGF receptor expression patterns can distinguish smooth muscle cell lineages (Lindahl et al., 1997). For example, the distal airway mesenchyme expresses high levels of PDGF-Ra, whereas proximal airway mesenchyme expresses relatively little. By contrast, both large vessel and capillary endothelium express PDGF-B, whereas both large vessel smooth muscle cells and capillary pericytes express high levels of PDGF-Rb. In addition, PDGF-A is essential for the differentiation of α-smooth muscle actin-expressing progenitor cells, which are ultimately responsible for parenchymal elastogenesis (Bostrom et al., 1996; Lindahl et al., 1997).

As with the regulation of epithelial cell differentiation, Wnt signaling contributes to mesenchymal development in the lung. A deficiency of Wnt7b results in severe abnormalities in lung mesenchymal tissue, particularly defects in the vascular smooth muscle (Shu et al., 2002). Additionally, Wnt5a can contribute to the regulation of lung Shh and FGF-10 expression, and it is necessary for the proper proliferation and/or differentiation of the distal mesenchyme (Li et al., 2002; 2005).

The pleura can also act as a source of factors that contribute to the regulation of lung mesenchymal cell proliferation and differentiation. FGF-9 is predominantly expressed in the lung pleura, and its absence leads to a severe reduction in lung mesenchyme development (Colvin et al., 2001). Likewise, RA is synthesized in the pleura (via Raldh2), and it appears to be capable of affecting distal lung morphogenesis during the canalicular stage of lung development (Malpel et al., 2000).

It is intuitive that the appropriate vascularization of the airspace is essential for efficient gas exchange. However, only recently has there been an appreciation for the relative importance of pulmonary vascular development and an understanding of the necessity of coordinated parenchymal capillary formation and secondary crest elongation during alveogenesis. The pulmonary vascular network arises through a combination of vasculogenesis, occurring proximal to conducting airways, and angiogenesis, arising from the aortic arches and the heart. There are two primary waves of vascularization during lung development: the first occurs at the saccular phase of development, and the second occurs during the alveolar phase.

Vascular endothelial growth factor (VEGF) is a master regulator of vascular development, and it appears to play a principal role in the regulation of the formation and maintenance of the lung vasculature and, thus, the alveoli. VEGF is expressed in both the mesenchyme and the epithelium during lung development (Acarregui et al., 1999) and it can promote the proliferation of endothelial as well as epithelial cells (Brown et al., 2001). The inhibition of VEGF function in adult rodents leads to a failure to maintain normal alveolar architecture and an emphysema-like phenotype (Kasahara et al., 2000; Petrache et al., 2005). Another molecule that seems to regulate vascular development in the lung is Scye1. Scye1, or EmapII, is a cytokine that is induced by apoptosis and that is shown to have tumor vasculature regulatory properties. Excess Scye1 inhibits vessel formation in an ectopic model of lung vascular development and leads to insufficient epithelial maturation (Schwarz et al., 2000).

Unfortunately, the further clarification of distinct mesenchymal cell lineages and the exploration of the mechanisms controlling their establishment are limited by the lack of specific markers, such as those that are available for distinct epithelial lineages (e.g., SPC, CCSP). However, optimism that significant progress will be made on this front in the near future seems warranted. In particular, the combination of genomics technologies currently available (including the power of genome-wide expression profiling to identify cell-type–specific markers) in combination with the power of mouse genetics to target specific cell populations should facilitate this endeavor.

V. SECONDARY CREST ELONGATION AND ALVEOGENESIS

Through the end of the saccular phase of lung development, the organ is woefully inefficient at performing its essential function: gas exchange. This is the result of two primary problems: a limited respiratory surface area and a poorly organized parenchymal capillary bed. Alveogenesis, which is the final stage of lung development, is the process that transforms the airspaces, primarily through the initiation and elongation of secondary septae (or crests) at points along the walls of the primary saccules. This is a highly coordinated and distinct morphogenic event, and its regulation is poorly understood. However, work during the past decade has identified some of the key pathways that contribute to the process. It is particularly important to point out that the molecules that regulate terminal lung development (both those related to insufficient maturation and those related to an inability to maintain and/or repair the lung) are likely to be more relevant to human disease. For example, mutations in the essential elastin fiber component fibrillin1 are associated with Marfan's-associated emphysema (Neptune et al., 2003).

It is clear that PDGF, FGF, and RA signaling play distinct but complementary and essential roles in the coordination of alveogenesis. As mentioned previously, PDGF-A is essential for the specification of a mesenchymal progenitor cell population that will play a prominent role in secondary crest elongation (Bostrom et al., 1996). These α-smooth muscle actin-expressing cells migrate to sites of potential secondary crest initiation during the saccular stage of development, and they contribute to secondary crest elongation at least in part by producing parenchymal elastic fibers. Coordinating appropriate parenchymal elastogenesis is a common feature of most pathways that are

known to play a primary role in alveogenesis. The requirement for proper elastogenesis in the establishment of appropriate airspace structure is highlighted by the failure of the lung to undergo alveogenesis in mice that are deficient in any one of many elastin fiber components (Wendel et al., 2000; Loeys et al., 2002; Neptune et al., 2003; Liu et al., 2004; Maki et al., 2005).

The development of the mammalian lung involves the repeated use of a limited number of regulatory modules (e.g., BMP-4, FGF-10) that reiteratively contribute to multiple developmental processes. The RA and FGF signaling pathways represent such regulatory modules, contributing to initial lung bud outgrowth, branching morphogenesis, and secondary crest elongation. Numerous lines of evidence reveal an important role for RA signaling in the establishment of proper alveolar structure. Deficiencies of numerous RA receptors, alone or in combination, lead to insufficient alveolar formation (Luo et al., 1996; Kastner et al., 1997; Massaro et al., 2000; McGowan et al., 2000; Massaro et al., 2003), and these deficiencies are almost always accompanied by decreased alveolar elastin production. In addition, supplementation with dietary RA can accelerate alveolar formation in neonatal rodents and promote alveolar regeneration and repair in adult rats (Massaro and Massaro, 1996; 1997; 2000). This is completely consistent with deficient alveogenesis in animals lacking one of numerous elastin fiber components, as described previously.

FGFR-3 and -4 coordinately promote alveogenesis as defined by the failure of terminal lung development in mice that are lacking the expression of both receptors (Weinstein et al., 1998; Hokuto et al., 2003). It is unclear at this time what regulatory mechanisms necessary for secondary crest elongation are affected in the absence of FGF signaling. Although it may seem counterintuitive, failed alveogenesis as a result of deficiencies in FGFR-3 and -4 is accompanied by hyperactive elastogenesis. This can be rectified with the numerous observations that insufficient elastin fiber formation is associated with incomplete alveolar formation if one models secondary crest elongation as a proximal–distal process in which RA and FGF act as competing morphogens to extend the secondary septum and occupy defined proximal and distal portions, respectively (Mariani, 2004; Mariani and Kaminski, 2004). This is referred to as the "balloon" model of alveogenesis (Figure 41.2), because it envisions the alveolus extending in a distal fashion away from the airways, whereas the proximal portion remains fixed in space, much like the inflation of a balloon. In this model, the tip of the alveolar/secondary septum occupies a proximal position and remains tethered to the conducting airways through extracellular matrix, whereas the base of the septum (which initially occupies a preproximal/predistal position) extends to eventually occupy a distal position. RA signaling, which typically specifies a proximal fate, occurs at the septal tip and promotes elastogenesis in a mesenchymal cell with a discrete phenotype. This is consistent with the normal accumulation of elastin fibers at the tips of alveolar septae, which essentially defines the "neck" or opening of the alveolus. Alternatively, FGF/FGFR signaling, which typically specifies a distal fate, occurs at the distalizing "base" of the septum and suppresses mesenchymal cells from assuming the proximal, elastogenic phenotype. Although this model accurately predicts the major observations regarding the regulation of alveogenesis, many aspects await experimental validation.

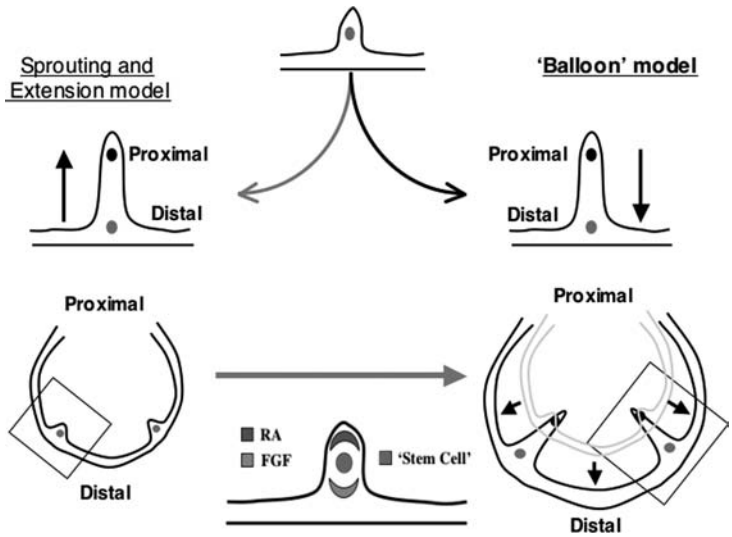

FIGURE 41.2 **The "balloon" model of alveogenesis. Top left,** The "sprouting and extension" model for secondary crest elongation depicts alveogenesis as secondary bud sprouting from the walls of primary septae and elongating into the airspace (Pierce and Shipley, 2000). **Top right,** The "balloon" model for the regulation of alveogenesis depicts secondary crest elongation as a distalization of the alveolar sac, with the tips of secondary crests being fixed in space by extracellular matrix tethering to the airways (Mariani, 2004). **Bottom,** This morphogenic process can be envisioned as occurring along a proximal–distal axis where alveolar saccules expand in a distal direction, like a balloon does when it is filled with air. A preproximal/predistal "stem cell" is differentiated into distinct proximal and distal cell populations. Proximalization is driven by retinoic acid signaling and characterized by elastin fiber production. Distalization is driven by fibroblast growth factor signaling, which functions to suppress the proximal phenotype. (See color insert.)

VI. TRANSITION TO AIR BREATHING

In addition to the tissue morphogenesis and cellular differentiation that occur throughout lung development, at birth, the organ must transition from being filled with fluid to filled with air. Although a significant body of work has identified discrete mechanisms that contribute to this transition (including the removal of water from the lumen and the secretion of surfactant), recent work has highlighted some regulatory control mechanisms for this transition and revealed that these are common genetic determinants of neonatal morbidity and mortality.

Pulmonary surfactant is composed of a complex mixture of lipids (both phospholipids and neutral lipids) and hydrophobic proteins that forms a physical barrier between airspace gas and surface liquid in the alveoli and that contributes a reduction in airspace surface tension. Surfactant is synthesized by type II pneumocytes, is stored in lamellar bodies and secreted during the antenatal period. Dynamic changes in the secretion, structure, and recycling of this lipoprotein complex during development, homeostasis, and lung disease are evident. For example, granulocyte macrophage colony stimulating factor is a critical regulator of surfactant recycling by alveolar macrophages, and a reduction in this activity leads to idiopathic alveolar proteinosis in humans (Trapnell et al., 2003).

During the past few years, Whitsett and colleagues have defined a regulatory network that controls the production of pulmonary surfactant, which is necessary for the transition to air breathing. It was initially discovered that one of the hydrophobic protein components of surfactant, Sftpb, was essential

for surfactant secretion. This was simultaneously appreciated in animal models of Sftpb deficiency and as genetic mutations in Sftpb were identified as a cause of respiratory distress in humans (Nogee et al., 1994; Clark et al., 1995). Similarly, a deficiency in the lamellar-body–associated membrane transporter Abca3 was identified as a basis for neonatal respiratory distress (Shulenin et al., 2004). Although the exact function of this transporter is not known, its association with lamellar bodies within type II pneumocytes suggests that it may participate in regulating surfactant organization and/or secretion. Subsequently, the forkhead box transcription factor, Foxa2, was identified as a master regulator of surfactant protein and Abca3 gene expression (Wan et al., 2004). Deficiency in Foxa2 in mice recapitulated many of the pathologic features of respiratory distress in humans. Recently, the transcription factor Cebpa was shown to contribute to this regulatory module. The deletion of Cebpa in mice leads to respiratory distress, structural deficiency in lung maturation, and alterations in surfactant and Abca3 protein expression (Martis et al., 2006). Cebpa is a direct target of both Foxa2 and Titf1, and it requires both of these transcription factors for appropriate expression.

A regulatory pathway involving Hif2a and VEGF has also been implicated in the regulation of surfactant production and secretion and the transition to air breathing (Compernolle et al., 2002). A deficiency of Hif2a in mice leads to respiratory distress, insufficient surfactant production, and reduced type II pneumocyte VEGF production (a known target of Hif2a). Blocking VEGF function resulted in respiratory distress in mice, and VEGF supplementation promoted surfactant production and was capable of promoting the survival of prematurely delivered mice. The significance of this pathway to lung maturation and surfactant production, how it may integrate with the one described previously, and its potential role in human disease are not known.

VII. CONCLUSION

Mammalian lung development is a complex process involving multiple morphogenic events that reliably result in an intricate, delicate, yet durable organ that is essential for life. Genetic studies of development in model organisms (particularly the mouse and the fruit fly) have clearly contributed to the global understanding of the mechanisms involved. Studies of these model organisms have further provided insight into the genetic and mechanistic nature of human lung diseases, such as neonatal respiratory distress syndrome. Although intensive research during the past decade has provided enlightenment concerning many of the regulatory mechanisms involved in creating this organ, much remains to be learned. We currently have a good understanding of the processes of early lung development (particularly branching morphogenesis), but we lack a thorough appreciation of the mechanisms that contribute to lung maturation, vascularization, and cell-type specification (particularly for nonepithelial cells). The recent advent of genomics-based technologies (including genome-wide expression profiling, conditional gene targeting, and whole-genome sequencing) promises to promote the rapid advancement of our current understanding. Given the potential to harness the knowledge of the regulation of lung development to both promote maturation in premature infants and to facilitate lung regeneration within the context of chronic lung disease, these future discoveries should prove to be most exciting.

SUMMARY

- Mammalian lung development is a highly complex and exquisitely regulated process involving morphogenic and regulatory processes that resemble vertebrate limb development and *Drosophila melanogaster* larval tracheal development.
- Lung development proceeds through histologically defined stages, each of which involves discrete morphologic characteristics, cellular alterations, and regulatory processes.
- Many of the signaling pathways that contribute to the individual stages and morphogenic processes of lung development also contribute to other processes at other times.
- We currently have a more thorough understanding of the regulation of early lung development, including branching morphogenesis, than we have of either lung maturation or the pathways that specify individual cell types (particularly with respect to mesenchymal lineages).
- Processes that contribute to terminal lung development, maturation, maintenance, and regeneration are more likely to be genetic determinants of human disease at least in part because failures early during the developmental process may be compounded over time.

ACKNOWLEDGMENTS

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GLOSSARY

Airspace
The respiratory portion of the lung, where gas exchange occurs. Sometimes referred to as *alveoli* (singular: *alveolus*). Airspace is distinct from the airways, which conduct air into and out of the lungs.

Alveogenesis/alveolization
The stage of lung development that encompasses the formation of the functional, respiratory portion of the organ. This process is initiated in humans during late fetal development, and it continues through the first few years of life. In rodents, this process occurs entirely postnatally, beginning at approximately 1 week of age.

Branching morphogenesis
A process of repetitive tube elongation and branching that occurs throughout early and mid lung development and that gives rise to the conducting airways.

Canalicular
The stage of lung development that is subsequent to the pseudoglandular stage and that involves the completion of the establishment of the conducting airways. During this stage, the lung is histologically rich in airways with clearly defined lumen. This stage occurs from weeks 16 to 26 of gestation in human development and from embryonic days 16.5 to 17.5 in mouse lung development.

Pseudoglandular
The initial stage of lung development, subsequent to organ budding from the ventral foregut, during which the lung histologically resembles a solid organ. A predominant feature of this stage is branching morphogenesis. This stage occurs from weeks 6 to 16 of gestation in human development and from embryonic days 10.5 to 16.5 in mouse lung development.

Saccular
The stage of lung development subsequent to the canalicular stage that involves the initiation of respiratory cell differentiation and airspace vascular development. It is histologically defined by the flattening of the epithelium at the distal end of the airways. This stage occurs from weeks 26 to 36 of gestation in human development and from embryonic day 17.5 to postnatal day 4 in mouse lung development.

Secondary crest elongation
One of the major morphologic changes that occurs during alveogenesis. It involves the formation and elongation of parenchymal tissue (secondary septae or crests) from locations along the walls of existing saccules (primary septae) in the distal respiratory region of the lung.

Vasculogenesis
The *de novo* formation of blood vessels distant from existing ones. It often involves either the reorganization of a disorganized vascular plexus and/or the transdifferentiation of cells/tissues into blood vessels.

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42
PANCREAS DEVELOPMENT AND STEM CELLS

MAUREEN GANNON

Departments of Medicine and Molecular Physiology and Biophysics, and Program in Developmental Biology, Vanderbilt University Medical Center, Nashville, TN

INTRODUCTION

Approximately 18 million Americans have diabetes, which is a heterogeneous group of disorders characterized by the decreased function of insulin-producing β cells and insufficient insulin output. Diabetes results from an absolute (type 1) or relative (type 2) inadequate functional β-cell mass. Whereas type 1 diabetes is characterized by the selective autoimmune destruction of β cells (Gale, 2001), type 2 diabetes occurs when the β-cell population fails to compensate for the increased peripheral insulin resistance associated with obesity (Kahn, 1998). Thus, both forms of the disease would greatly benefit from treatment strategies that could enhance β-cell regeneration and/or proliferation. Although there have been some encouraging results from islet transplantation in achieving the remission of type 1 diabetes (Shapiro et al., 2000; Ryan et al., 2001), the limited amount of donor tissue obtainable makes this potential treatment unavailable to most patients. The ability to induce β cells or whole islets from pancreatic stem cells *in vivo* or *in vitro* or embryonic stems *in vitro* would provide an alternative source of transplantable tissue (Lumelsky et al., 2001; Odorico et al., 2001; Bonner-Weir and Sharma, 2002). Additionally, studies addressing the proliferation, regeneration, and neogenesis of β cells in the adult pancreas could lead to the restoration of β-cell mass in individuals with type 1 diabetes and enhanced β-cell compensation in patients with type 2 diabetes. Successful and reliable methods of generating islet endocrine cells *in vivo* or *in vitro* will benefit greatly from a thorough understanding of the normal developmental processes that occur during pancreatic organogenesis (e.g., transcription factors, cell-signaling molecules, and cell–cell interactions that regulate endocrine proliferation and differentiation from the embryonic pancreatic epithelium).

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Recently, much progress has been made in the identification of the factors involved in the normal development and differentiation of the various pancreatic cell types. Interestingly, mutations of many of these developmentally important factors have been identified in individuals with diabetes. This chapter will summarize what is known about the regulation of pancreas development and about the factors that control mature islet function. We will also discuss potential pancreatic and endocrine stem/progenitor cell sources and the recent progress in generating insulin-producing cells in culture.

I. THE INITIAL STAGES OF PANCREATIC BUD FORMATION

The mature pancreas is composed of two distinct functional units: the exocrine component of the pancreas, which consists of clusters of acinar cells that produce and secrete digestive enzymes such as amylase and elastase, and the ductal network, which transports the acinar secretions into the rostral duodenum. The endocrine compartment is composed of spherical clusters of at least five hormone-producing cell types: insulin (β cells), glucagon (α cells), somatostatin (δ cells), ghrelin (ε cells), and pancreatic polypeptide (PP cells; Figures 42.1 and 42.2). These endocrine clusters comprise microorgans known as the *islets of Langerhans*. Together, these islet hormones regulate glucose homeostasis by facilitating the uptake of ingested glucose into cells and stimulating glucose production by the liver during times of fasting. Acinar, ductal, and endocrine cells are all derived from the endoderm during embryonic development (Percival and Slack, 1999).

FIGURE 42.1 **Schematic of pancreas and islet development. A,** The pancreas arises as dorsal and ventral evaginations from the posterior foregut endoderm on embryonic day 9.5, which is marked by the expression of the *Pdx1* transcription factor *(yellow)*. Markers of the early pancreatic buds include the transcription factors *Ptf1a* and *Hb9 (blue)*. Within the developing buds, a subset of cells expresses markers of the endocrine lineage, including *Ngn3, Isl-1,* and *Pax6 (red)*. **B,** As development proceeds, the pancreatic epithelium *(yellow)* becomes a highly branched ductal network. Endocrine cells *(green nuclei)* and exocrine cells *(blue nuclei)* arise from the ducts. Endocrine progenitors are scattered throughout the epithelium, and they express *Ngn3 (red nuclei)*. These cells maintain *Ngn3* expression as they delaminate from the epithelium *(tan-colored cells)*. *Ngn3* is downregulated as hormone expression begins *(green, red, and orange cells)*, and more general endocrine markers such as *Isl-1* and *Pax6* are expressed *(green nuclei)*. **C,** Mature islets begin to form during late gestation. In the mouse, insulin-producing β cells *(green)* are found at the islet core, and all other hormone-producing cells are located at the periphery. (See color insert.)

The epithelial component of the pancreas arises from dorsal and ventral outgrowths of the posterior foregut endoderm just caudal to the liver diverticulum in all vertebrates examined, including humans (see Figure 42.1; Wessels and Cohen, 1967; Kim et al., 1997; Kelly and Melton, 2000; Field et al., 2003; Ober et al., 2003). The dorsal and ventral buds later fuse to form a single organ (this occurs on embryonic day 12.5 in the mouse). Pancreatic bud formation can be observed as early as day 25 of gestation in humans (Ashraf et al., 2005), on embryonic day 9.5 in the mouse (Slack, 1995), and 24 hours postfertilization in zebrafish (see Figure 42.2; Field et al., 2003). In mammals, frogs, and chickens, both pancreatic buds generate exocrine and endocrine cells. By contrast, in zebrafish, the posterior (ventral) bud generates the endocrine tissue, which usually consists of a single islet, whereas the anterior (dorsal) bud gives rise primarily to the pancreatic duct and the acinar cells, although endocrine cells do arise from dorsal bud derivatives later during development (Field et al., 2003).

The morphogenesis of the pancreatic epithelium yields a highly branched ductal network within which multipotent progenitors for both exocrine and endocrine cells are thought to reside (see Figure 42.1; Bouwens and De Blay, 1996; Fishman and Melton, 2002). It is unclear whether there ever exists a common progenitor cell that is capable of giving rise to all of the different cell types within the pancreas or whether specific types of progenitors with a more limited potential (i.e., endocrine, exocrine, duct) already exist at the earliest stages of pancreas development. There is currently a lack of markers that

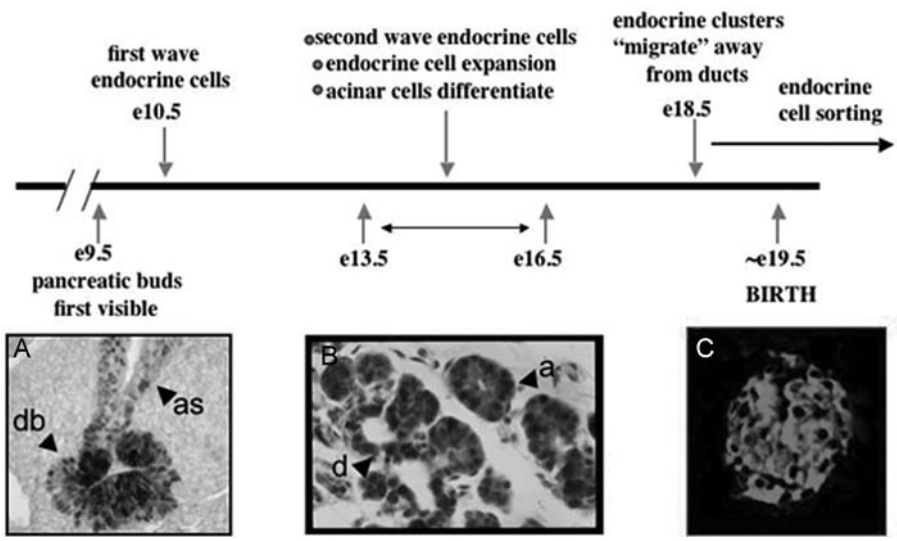

FIGURE 42.2 **Timeline of pancreas development in the mouse. Key events in mouse pancreas development are shown. A,** Pancreatic bud evagination can first be detected on embryonic day 9.5. In this image, the *Pdx1* expression domain is marked by the brown nuclei, and it includes the antral stomach *(as)* and the dorsal pancreatic bud *(db)*, which is also marked by a pancreas-specific lacZ transgene *(blue)*. Endocrine differentiation occurs in two waves. The first begins on embryonic day 10.5 and extends to embryonic day 13.5. The second wave begins on embryonic day 13.5 and continues until neonatal stages. **B,** Acinar gene expression *(a; amylase in brown)* begins on embryonic day 14.5. Both acinar cells and endocrine cells bud off of the ductal epithelium *(d; blue)*. **C,** During late gestation, endocrine cells cluster, migrate away from the ductal epithelium, and organize into islets with β cells at the core and other hormone cell types at the periphery. *Green,* Insulin, *red,* glucagon. (See color insert.)

identify progenitor cells within the pancreas akin to those that are known to be in the hematopoietic field, although several laboratories are using genome expression profiling at different stages of pancreas development in an attempt to identify such markers (Chiang and Melton, 2003; Wells, 2003; Gu et al., 2004). Lineage-tracing studies have determined, however, that all pancreatic cell types arise from a cell that expresses the pancreatic duodenal homeobox 1 *(pdx1)* and *Pft1a/p48* transcription factors (discussed later; Gu et al., 2002; Kawaguchi et al., 2002).

The expression of endocrine hormones such as glucagon and insulin is detected even at early pancreatic bud stages (embryonic day 10.5); exocrine-specific gene transcription does not commence until embryonic day 14.5 (see Figure 42.2; Gittes and Rutter, 1992). Pancreatic endocrine differentiation actually occurs in two waves during embryogenesis (see Figure 42.2; Pictet et al., 1972; Pang et al., 1994; Prasadan et al., 2002). The first wave occurs between embryonic days 9.5 and 13.5. Unlike second-wave endocrine cells, these early differentiating hormone-producing cells can develop in the absence of the critical pancreatic transcription factor *pdx1* (Ahlgren et al., 1996; Offield et al., 1996); they lack other genetic markers of mature islet endocrine cells (Pang et al., 1994; Lee et al., 1999; Wilson et al., 2002), and they have been shown by lineage-tracing analyses to not contribute to mature islets (Herrera et al., 1994; Herrera, 2000). During the second wave of endocrine differentiation, which commences on embryonic day 13.5 in the mouse, the numbers of endocrine cells greatly increase. These endocrine cells go on to populate the mature islets. The mechanism for the increase in endocrine cells at these stages is unknown. The formation of mature, optimally functional islets requires the generation of appropriate numbers of each endocrine cell type, and this process is likely regulated by positive and negative factors that influence cell proliferation and differentiation.

In a process that is very reminiscent of neurogenesis in Drosophila and other organisms, islet organogenesis involves the delamination of specified endocrine cells from the ductal epithelium, detachment from the ducts, and the formation of adherent islet clusters (see Figure 42.1; reviewed by Edlund, 2001). As in Drosophila, the specification of endocrine progenitors within the ductal epithelium is dependent on cell–cell communication and lateral inhibition using the Notch signaling pathway (Apelqvist et al., 1999; Jensen et al., 2000; Murtaugh et al., 2003). In the developing pancreatic ductal epithelium, cell–cell interactions involving Notch–Delta signaling determine which cells will initiate the endocrine genetic program by activating *Ngn3* expression (Apelqvist et al., 1999; Jensen et al., 2000). Cells expressing higher levels of Notch signaling remain within the epithelium and actively repress *Ngn3* expression, whereas those cells in which Delta levels become elevated activate *Ngn3,* exit from the epithelium, and ultimately give rise to the endocrine population (see Figure 42.1; Gu et al., 2002). In the developing human pancreas, cell surface proteins, including a cell adhesion molecule and certain integrins, have been identified that may mark endocrine progenitor cells within and delaminating from the ductal epithelium (Cirulli et al., 1998, 2000). After delamination, the endocrine cells begin to organize into clusters that are initially still located close to ducts (see Figure 42.1). On embryonic day 18.5, these clusters begin to lose their proximity to the ductal epithelium, they become surrounded by exocrine tissue, and they form mature islets (see Figures 42.1 and 42.2). As islets form, the endocrine cells segregate such that, in mice,

the insulin-producing β cells are located at the core, and glucagon-, somatostatin-, ghrelin- and PP-producing cells are located at the periphery or mantle (see Figures 42.1 and 42.2). Little is known about how the different endocrine cell types and their precursors interact with one another to form functional islets. The processes of endocrine delamination and islet formation likely include changes in the expression of lineage-specific transcription factors, cell adhesion molecules, and extracellular matrix components.

II. INDUCTIVE INTERACTIONS DURING PANCREAS DEVELOPMENT

Pancreas development is dependent on an interaction between epithelial (endodermal) and mesodermal components (Edlund, 2002; Kim and MacDonald, 2002; Kumar and Melton, 2003; Wilson et al., 2003). Signals from the notochord have been implicated in pancreas specification and outgrowth (Kim et al., 1997; Hebrok et al., 1998, 2000; Kim and Melton, 1998), whereas pancreatic mesenchyme stimulates the growth of the endodermal epithelium (Wessels and Cohen, 1967; Ahlgren et al., 1997). In turn, the endoderm influences the character of the overlying mesoderm (Slack, 1995; Apelqvist et al., 1997).

A. Early Inductive Events in Pancreatic Endocrine Differentiation: The Role of the Notochord

Wessels and Cohen (1967) suggested that signals derived from dorsal axial tissue such as the notochord might be involved in inducing the outgrowth of the dorsal pancreatic bud. The notochord transiently contacts the endodermal epithelium directly in the region from which the dorsal pancreatic bud will form during stages that occur before pancreatic bud outgrowth. Experimental manipulations in chick embryos revealed that, in the absence of the notochord, the dorsal pancreatic bud undergoes only limited outgrowth and branching, and it fails to activate the expression of pancreatic transcription factors (e.g., *pdx1, Isl-1, Pax6*) and of markers of differentiated endocrine or exocrine cells (Kim et al., 1997). By contrast, the outgrowth and differentiation of the ventral pancreatic bud occurs normally in the absence of the notochord. It is currently unclear what tissue interactions promote ventral pancreas development, although genes involved in ventral bud development have been identified (discussed later). Activin βB and fibroblast growth factor (FGF)-2 are likely to be the endogenous notochord-derived signals that induce dorsal pancreas bud outgrowth and differentiation (Hebrok et al., 1998). One of the main functions of notochord-derived factors seems to be the repression of endodermal Sonic hedgehog (Shh) expression in the region that is destined to give rise to the pancreas (Hebrok et al., 1998). Shh is expressed throughout the embryonic gut endoderm with the exception of the dorsal and ventral pancreatic bud endoderm. The transplantation of an ectopic notochord to non-pancreatic regions of the developing gut tube results in decreased Shh expression in the region that is adjacent to the transplant (Hebrok et al., 1998). The maintenance of Shh in the pancreatic field using a transgenic approach results in the impaired development of the pancreatic epithelium and the altered character of the overlying mesoderm such that it expresses markers that are consistent with small intestine smooth muscle (Apelqvist et al., 1997).

B. Early Inductive Events in Pancreatic Endocrine Differentiation: The Role of Endothelial Cells

The vasculature of the pancreas is derived from the mesodermal germ layer. Although islets represent only approximately 2% of the total mass of the pancreas in an adult, they receive up to 15% of the blood flow (Lifson et al., 1980, 1985), likely as a result of their role as endocrine organs secreting hormones directly into the bloodstream. The morphology and architecture of endothelial cells differs among the different capillary beds (LeCouter et al., 2002). Vessels are classified as continuous, fenestrated, or discontinuous. Capillaries in skeletal muscle, heart, lung, and brain have a continuous endothelium, whereas capillaries in endocrine glands such as the islets are fenestrated (LeCouter et al., 2002; Cleaver and Melton, 2003). Thus, in addition to producing angiogenic stimuli for inducing the ingrowth of new vessels, tissues provide factor(s) to direct the differentiated phenotype of the endothelium. For example, early differentiating pancreatic endocrine cells (both first- and second-wave cells) produce angiogenic factors including vascular endothelial growth factor (VEGF) and angiopoietin 1 (Brissova et al., 2006); the expression of these factors is maintained in adult islets (Christofori et al., 1995; Rooman et al., 1997; Lammert et al., 2001). The continued expression of these factors in adult islets suggests that the maintenance of a fenestrated endothelium is critical for mature islet function. In addition to islet endocrine cells communicating with vascular endothelial cells via secreted growth factors, endothelial cells have also been shown to signal to the pancreatic epithelium, thus influencing the differentiation of endocrine cells (Lammert et al., 2003a, 2003b; Yoshitomi and Zaret, 2004).

Pancreatic bud outgrowth is initiated at sites in the posterior foregut endoderm, where it contacts the endothelium of major blood vessels; endocrine differentiation initially occurs in cells that have direct contact with endothelial cells (Lammert et al., 2001). On embryonic day 10.5, insulin expression is detected at sites at which the dorsal pancreatic bud contacts portal vein endothelium. The importance of vascular endothelial cells in pancreatic endocrine differentiation has been demonstrated both in tissue recombination experiments and in genetically modified mice. For example, embryonic day 8.5 endoderm cultured in the absence of endothelial cells failed to activate either Pdx1 or insulin protein expression, whereas, when undifferentiated endoderm was cultured in combination with dorsal aorta, both Pdx1 and insulin were induced (Lammert et al., 2001). The examination of VEGF receptor type 2 (VEGFR-2/flk-1) null mutant mice, which die before the second wave of endocrine differentiation, revealed that early insulin- and glucagon-positive cells fail to develop (Lammert et al., 2001; Yoshitomi and Zaret, 2004). These mice express most pancreatic/endocrine transcription factors *(pdx1, hnf6, Ngn3, NeuroD, Prox1,* and *Hb9)*, with the exception of the early pancreatic bud marker, *ptf1a* (Yoshitomi and Zaret, 2004). Taken together, these data provide strong support for an endothelial-derived endocrine-inducing factor (or factors). Although the identity of this factor is currently unknown, it follows that, if endothelial cell numbers were to increase, the amount of the inducer (and thus the amount of endocrine cells) would also increase. To this end, the Melton laboratory generated transgenic mice expressing $VEGF_{164}$ throughout the entire pancreatic bud early during development (Lammert et al., 2001). These transgenic embryos showed greatly increased

vasculature in the pancreas and a corresponding threefold increase in islet number and islet area. Ectopic insulin expression was found adjacent to the ectopically induced endothelial cells.

The evidence is increasing that, during both liver and pancreas development, endothelial cells produce an instructive signal that induces differentiation and morphogenesis (Lammert et al., 2001; Matsumoto et al., 2001; Yoshitomi and Zaret, 2004). What might this factor be? Vascular endothelial cells are known to produce several different secreted growth factors, including FGF, transforming growth factor (TGF)-β, Wnt, and hepatocyte growth factor (HGF; Lammert et al., 2003a). In the liver, a VEGFR-1/flt-1 receptor-specific agonist causes endothelial cells to express HGF in a paracrine fashion. HGF has been shown to be mitogenic to β cells (Otonkoski et al., 1994; Hayek et al., 1995; Garcia-Ocana et al., 2000). Thus, in pancreatic endothelial cells, VEGF signaling through VEGFR-1 may induce the expression of HGF, which in turn promotes endocrine proliferation. These studies highlight the reciprocal communication between pancreatic endocrine cells and endothelial cells.

III. GENES THAT AFFECT PANCREATIC BUD DEVELOPMENT

Pancreatic development requires factors that act autonomously within the endodermally derived epithelium as well as factors that function within the adjacent associated mesenchyme. Gene inactivation in mice has identified transcription factors that affect the differentiation of all or of a subset of the pancreatic cell types. Factors that regulate the differentiation and function of islet β cells are candidates for susceptibility genes in type 2 diabetes. Indeed, most of the genetic lesions that result in a dominant form of the disease called maturity onset diabetes of the young (MODY) are associated with mutations in transcription factors that are expressed in adult β cells: *HNF4α* (MODY1), *HNF1α* (MODY3), *pdx1* (MODY4), *HNF1β* (MODY5), and *Beta2/NeuroD* (MODY6; Hattersley et al., 2000). Some of these factors play critical roles in distinct stages of pancreatic/endocrine development, including pancreatic bud outgrowth and branching, endocrine differentiation, and/or mature islet function (Gannon and Wright, 1999; Edlund, 2001; Wilding and Gannon, 2004). Promoter analysis of islet-specific genes such as insulin has also helped to identify *trans*-acting factors that are critical for normal pancreas and/or endocrine development (Madsen et al., 1997; Sander and German, 1997; Edlund, 1998). By understanding how these different transcription factors function during the normal pathway of islet differentiation, one may be able to manipulate this pathway to induce the differentiation of pancreatic stem cells *in vivo* or to influence the production of functional β cells and/or islets from pancreatic or embryonic stem cells *in vitro* (Edlund, 2002). This section will summarize some of the results from gene inactivation and/or over-expression studies to determine the role of these factors in pancreas development, endocrine differentiation, and mature β-cell function.

A. Endodermally Expressed Genes That Affect Pancreatic Bud Formation

1. Pdx1

The homeodomain transcription factor *pdx1 (Ipf1)* is one of the earliest known markers of the developing pancreas (see Figures 42.1 and 42.2; Gannon

and Wright, 1999), and lineage-tracing analysis reveals that all cells within the endodermal component of the pancreas are derived from a *pdx1*$^+$ cell (Gu et al., 2002). *pdx1* is expressed early throughout the endoderm of the pancreatic buds as well as by the antral stomach, the rostral duodenum, and the common bile duct. However, it becomes highly enriched in insulin-producing β cells during late gestation, with lower levels of expression found in some acinar cels (see Figure 42.1; Guz et al., 1995; Offield et al., 1996; Wu et al., 1997). Pdx1 binds to and activates the insulin promoter and other β-cell–specific genes such as GLUT2, glucokinase, and islet amyloid polypeptide (Figure 42.3). In both mice and humans, the homozygous inactivation of *pdx1* results in pancreatic agenesis, whereas heterozygotes have impaired β-cell function and are prone to diabetes (Jonsson et al., 1994; Offield et al., 1996; Stoffers et al., 1997, 1998; Ahlgren et al., 1998; Dutta et al., 1998). Mutations in *pdx1* have also been identified in some patients with type 2 diabetes (Stoffers et al., 1997). In *pdx1* null mouse mutants, the dorsal bud undergoes limited proliferation and outgrowth to form a small, irregularly branched ductule (Offield et al., 1996). Transient insulin$^+$ cells and longer-lived glucagon$^+$ clusters are found in the mutant epithelium (Ahlgren et al., 1996; Offield et al., 1996), and these may represent first-wave (immature) endocrine cells. Thus, *pdx1* is not absolutely essential for insulin or glucagon expression. *pdx1* inactivation specifically in adult β cells results in type 2 diabetes, thus demonstrating a role for *pdx1* in the maintenance of β-cell function (Ahlgren et al., 1998).

2. Ptf1a

Ptf1a (p48) is the pancreas-specific component of the heterotrimeric basic helix–loop–helix (bHLH) complex pancreas transcription factor 1 (PTF1; Cockell et al., 1989; Krapp et al., 1996). The two other components of the PTF1 complex are the constitutively expressed HeLa E-box binding factor (Cockell et al., 1989), and the mammalian Suppressor of Hairless (RBP-J; Obata et al., 2001) or its paralog RBP-L (Beres et al., 2006). Although it was first identified as a regulator of exocrine-specific genes (Krapp et al., 1996; Rose et al., 2001), PTF1 was subsequently shown to be essential early

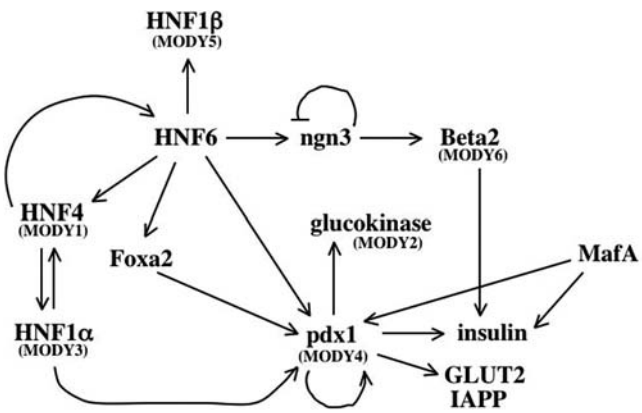

FIGURE 42.3 **Simplified pancreas transcription factor network.** Some of the factors that are important in the specification of the endocrine lineage and subsequent differentiated β cells are shown. In particular, the interactions between different Maturity onset diabetes of the young genes, transcriptional targets of Hnf6 and Pdx1, and transcription factors that transactivate the insulin promoter are highlighted. Arrows indicate direct transcriptional targets.

during development for normal endocrine and exocrine pancreas formation in both mice and humans (Krapp et al., 1998; Obata et al., 2001; Kawaguchi et al., 2002; Sellick et al., 2004). Mice lacking *Ptf1a* have no detectable ventral bud outgrowth; the dorsal bud initiates but then arrests as a duct-like structure that lacks differentiated acinar cells (Krapp et al., 1998; Kawaguchi et al., 2002). Small endocrine clusters that contain insulin and glucagon cells are present within this structure, and a few isolated hormone$^+$ endocrine cells can be detected within the spleen. These may represent first-wave endocrine cells. The results of *Ptf1a* gene inactivation were supported by analyses showing that *Ptf1a* expression is detected as early as embryonic day 9.5 throughout the developing pancreatic buds (see Figure 42.1; Kawaguchi et al., 2002). Lineage-tracing studies further revealed that acinar, ductal, and endocrine cells are all derived from a *Ptf1a*-expressing cell (Kawaguchi et al., 2002). In zebrafish, *Ptf1a* is required for the endocrine and exocrine tissue that arises from the anterior (dorsal) bud, but it is not required for posterior (ventral) bud formation and outgrowth, which differentiates into endocrine tissue (Lin et al., 2004).

3. Hlxb9

Hlxb9 is the gene that encodes the Hb9 homeodomain protein, which is prominently expressed in adult islet β cells. Hb9 is expressed transiently throughout the prepancreatic epithelium during the early stages of mouse pancreatic development (embryonic days 8 to 10.5) in both the dorsal and the ventral anlagen coincident with *Pdx1* and *Ptf1a* expression (see Figure 42.1), and it is reexpressed later (on embryonic day 17.5) in differentiated β cells (Li et al., 1999). In the absence of Hlxb9 expression, the dorsal bud of the pancreas fails to develop (Harrison et al., 1999; Li et al., 1999). By contrast, the ventral pancreatic endoderm develops, and it forms both endocrine and exocrine tissues; however, the endocrine cells within the islets are disorganized, and they have reduced numbers of insulin-producing cells that demonstrate the reduced expression of some markers of terminal β-cell differentiation.

4. Hex

The *Hex* homeobox gene is expressed in the ventral posterior foregut in the region that will give rise to both the liver bud and the ventral (but not the dorsal) pancreatic bud. The examination of *Hex* null embryos reveals a requirement for Hex in the proliferation and outgrowth of the liver bud and in the specification of the ventral pancreas (Bort et al., 2004). In the absence of Hex, the ventral pancreatic bud does not form, and it fails to express *Pdx1, Ptf1a,* and *Hlxb9*. Dorsal pancreas specification appears to be normal in these mutants. Interestingly, explants of presumptive ventral pancreas endoderm differentiate normally in the absence of *Hex,* expressing *Pdx1* and endocrine lineage markers such as *Isl-1, Ngn3*, and *Beta2*. These results suggest that *Hex* null endoderm is fully competent to differentiate according to its normal fate but that it is susceptible to influences from surrounding tissues within the intact embryonic environment that prevent ventral pancreas specification and differentiation. Indeed, when cocultured with cardiogenic mesoderm, $Hex^{-/-}$ ventral endoderm no longer expresses any pancreatic markers (Bort et al., 2004).

B. Mesodermally Expressed Genes that Affect Pancreatic Bud Formation

1. Isl-1

The LIM homeodomain transcription factor *Islet-1 (Isl-1)* was identified as a factor that binds to and transactivates the insulin gene promoter. It is expressed in all islet endocrine cell types in the embryo (see Figure 42.1) and the adult and in the mesodermally derived mesenchyme surrounding the dorsal (but not the ventral) pancreatic bud (Ahlgren et al., 1997). Similar to the phenotype found in *Hlxb9* mutant pancreata, the dorsal pancreatic bud does not develop and differentiate in *Isl-1* null embryos (Ahlgren et al., 1997). The ventral bud grows normally, but it fails to produce any endocrine hormone$^+$ cell types; acinar cell differentiation appears to be normal. Unlike *Hlxb9* mutant pancreata, in which the dorsal mesenchyme remains intact, the absence of dorsal bud derivatives in *Isl-1* null embryos is specifically the result of the loss of dorsal mesenchyme, because mesenchyme from wild-type embryos can rescue the differentiation of exocrine cells from *Isl-1* mutant dorsal pancreatic endoderm in culture. Importantly, hormone$^+$ cells are not rescued by wild-type mesenchyme, which suggests that *Isl-1* has a non–cell-autonomous role in the mesenchyme for dorsal bud outgrowth and a cell-autonomous role in the endoderm for endocrine differentiation. These results underscore the requirement for the pancreatic mesenchyme in pancreas development.

2. N-cadherin

During the early stages of pancreas development (embryonic days 9.0 to 12.5), the cell adhesion molecule N-cadherin is expressed in both the pancreatic epithelium and the surrounding mesenchyme. After embryonic day 12.5, expression becomes restricted to the formation of clusters of endocrine cells. In embryos that lack N-cadherin, the dorsal pancreatic bud fails to form, although genes such as *Hlxb9* and *Isl-1* are expressed normally, and Shh expression is repressed in the dorsal endoderm (Esni et al., 2001). N-cadherin function is not required within the pancreatic endoderm, because coculture with wild-type mesenchyme can rescue branching morphogenesis, exocrine, and endocrine differentiation. In addition, restoring cardiac and circulatory function in N-cadherin null mice by the cardiac-specific transgenic expression of N-cadherin rescues the formation of the dorsal pancreas (Edsbagge et al., 2005). On the basis of this observation, it was proposed that soluble factors present in plasma are critical for the formation and/or maintenance of the dorsal pancreatic mesenchyme. It was found that sphingosine 1-phosphate present in plasma promotes the budding of the pancreatic endoderm by stimulating pancreatic mesenchymal cell proliferation and that sphingosine 1-phosphate receptors are located within the mesenchyme (Edsbagge et al., 2005).

IV. GENES THAT AFFECT THE DIFFERENTIATION OF PARTICULAR PANCREATIC CELL TYPES

A. Genes Involved in Exocrine Differentiation

There is actually very little known about factors that act to specify the exocrine cell population from progenitors within the ductal epithelium. It was previously thought that cells that fail to activate Notch became endocrine

cells, whereas those that activate the Notch pathway became exocrine cells. It is now clear that Notch activation maintains cells in a proliferative, progenitor state and that other signals are required to generate acinar cells or endocrine cells from this pool. The activation of Notch has recently been shown to inhibit exocrine differentiation in both the mouse and the zebrafish (Hald et al., 2003; Murtaugh et al., 2003; Esni et al., 2004).

1. Pdx1 and Ptf1a

Despite the severe defects in pancreas development that occur in the absence of either *Pdx1* or *Ptf1a*, there are some differentiated endocrine cells detected in both mutants. By contrast, there is a complete absence of exocrine tissue in both the *Pdx1* and *Ptf1a* null mutant animals, which highlights the fact that these genes are essential for exocrine development. As mentioned previously, *Ptf1a* is highly enriched in acinar cells after embryonic day 13.5, and the PTF1 heterotrimeric transcription factor complex has been shown to bind to several exocrine-specific gene promoters (Krapp et al., 1996; Rose et al., 2001). The loss of RBP-J, which is another component of the PTF1 complex, results in the accelerated differentiation of endocrine cells (Apelqvist et al., 1999), thus supporting the idea that a functional PTF1 is required for acinar differentiation. *Pdx1,* although it is expressed at much lower levels in mature acinar cells as compared with β cells, has also been found to regulate acinar gene expression in cooperation with *Pbx1* and *Meis2* (Swift et al., 1998). The maintenance of *pdx1* expression in the exocrine lineage during embryonic development is required for acinar differentiation (Hale et al., 2005).

2. Mist1

The *Mist1* bHLH transcription factor is expressed in acinar cells, and it activates the acinar genes that are involved in gap junction communication and coordinated exocytosis. *Mist1* null mutant mice exhibit the extensive disorganization of exocrine tissue and defects in the regulated exocytosis pathway, which results in inappropriate intracellular enzyme activation. These changes mimic those observed in pancreatic injury, such as those seen with chronic pancreatitis. Thus, it has been proposed that *Mist1* is a key regulator of acinar cell function, stability, and identity (Pin et al., 2001; Rukstalis et al., 2003; Johnson et al., 2004).

B. Genes Involved in General Endocrine Specification and the Differentiation of Particular Lineages

Although all islet endocrine cells express some common factors that promote endocrine specification and differentiation, it is likely that each particular islet endocrine cell type is specified by a different combination of transcription factors. Many of the lineage-restricted or lineage-specific transcription factors are actually expressed more broadly in the pancreatic epithelium or in the endocrine population early during development; they become gradually restricted as development proceeds to refine the pattern of gene expression to what is observed in the adult islet. Gene-expression and mutational analyses in mice have proven to strongly correlate with gene function in humans, because mutations in many of the genes that will be discussed later have been identified in individuals with type 2 diabetes, including *Pdx1, Pax6,* and *Beta2*.

1. Prox1

The homeobox gene *Prox1* is expressed in the posterior foregut endoderm in the presumptive pancreas region before bud outgrowth (Burke and Oliver, 2002), and it has been shown to be essential for normal liver bud outgrowth (Sosa-Pineda et al., 2000). On embryonic day 13.5, *Prox1* is expressed in most cells throughout the pancreatic epithelium. As the second wave of endocrine differentiation commences after embryonic day 13.5, *Prox1* becomes more highly expressed in $NGN3^+$ (a marker of committed endocrine progenitor) and *Isl-1*$^+$ (a marker of all islet endocrine) cells, and it is downregulated in differentiating acinar cells. After birth, *Prox1* expression is maintained at high levels in the ductal epithelium and in peripheral islet cell types, with lower levels found in β cells. On embryonic day 15.5 (the time at which *Prox1*-deficient embryos die as a result of complications in other organ systems), the *Prox1* mutant pancreatic epithelium is less branched than that of wild type, and it contains many fewer endocrine cells (Wang et al., 2005). By contrast, the number of differentiated acinar cells is relatively increased, and the pancreas has increased levels of *Ptf1a* with decreased levels of markers of endocrine lineage (e.g., *Ngn3*). Thus, *Prox1* may be required within a bipotential acinar/endocrine pancreatic progenitor to promote differentiation down the endocrine lineage.

2. HNF6

Hepatic nuclear factor 6 *(HNF6/Onecut-1)*, as the name implies, was first identified in the liver, but it is actually more broadly expressed in the developing endoderm. Target genes for HNF6 include *Foxa2, Pdx1* (MODY4), and *HNF4* (MODY1), which are critical endodermal regulators: *Foxa2* is involved in the β-cell–specific expression of *Pdx1,* and *HNF4* activates *HNF1α* (MODY3). In turn, HNF1α activates *Pdx1,* and *HNF4* regulates *HNF6* (see Figure 42.3; reviewed by Jensen, 2004). Thus, alterations in the expression of a single HNF or MODY gene can affect the expression of multiple genes in this hierarchy. One of the interesting things about *HNF6* is its dynamic expression pattern with the pancreas. Similar to *Pdx1* and *Ptf1a, HNF6* is initially expressed throughout the early pancreatic epithelium. As development proceeds, *HNF6* is maintained in the ductal epithelium and in acinar cells, but it becomes downregulated—specifically in endocrine cells by embryonic day 18.5 (Landry et al., 1997; Rausa et al., 1997). The decreased expression of *HNF6* in islet endocrine cells coincides with islet morphogenesis and β-cell maturity in preparation for birth (see Figure 42.2). This downregulation is critical for normal islet ontogeny and function: continued *HNF6* expression in islets impairs the separation of endocrine cells from the ductal epithelium, disrupts the organization of endocrine cell types within the islet (core vs. mantle), and severely compromises β-cell physiology, thus leading to overt diabetes (Gannon et al., 2000; Tweedie et al., 2006).

Despite its early broad expression pattern in the pancreatic buds, *HNF6* function is not required to generate a pancreas. In the absence of *HNF6, Pdx1* gene activation and pancreas bud outgrowth are delayed, and this results in a slightly hypoplastic pancreas at birth (Jacquemin et al., 2003). *HNF6* is an upstream activator of *Ngn3* (see Figure 42.3; Jacquemin et al., 2000), which is a transcription factor that is expressed in endocrine precursors, and it is a transcriptional target of the Notch–Delta signaling pathway.

Ngn3$^{-/-}$ mice lack endocrine cells in both the pancreas and the small intestine (Gradwohl et al., 2000). *HNF6*$^{-/-}$ mice have decreased numbers of *Ngn3*$^{+}$ cells during embryogenesis, and they lack islets at birth (Jacquemin et al., 2000). Islets do develop later, but they are abnormal, and the mice are glucose intolerant. The presence of islets in the absence of *HNF6* suggests that other factors partially compensate for *HNF6,* and, indeed, other closely related factors are also expressed in the developing pancreas (Jacquemin et al., 2003). *HNF6* function is therefore required to generate endocrine progenitors in the appropriate numbers.

3. Ngn3/NeuroD

There are two closely related bHLH transcription factors that are involved in the early stages of pancreatic endocrine development: *Ngn3* and *Beta2/NeuroD* (MODY6). *Ngn3*-expressing cells are first detected scattered throughout the pancreatic epithelium on embryonic day 9.5. Their numbers reach a peak on embryonic day 15.5 and then decrease to nearly undetectable levels by birth (Gradwohl et al., 2000). Ngn3^{+} cells are found within or adjacent to the ductal epithelium, and they do not coexpress any of the islet endocrine hormones (see Figure 42.1). In the absence of *Ngn3,* endocrine markers within the pancreas (including both broad and lineage-restricted transcription factors and all hormones) are missing (Gradwohl et al., 2000). Thus, *Ngn3* represents the only known gene that specifically marks the endocrine progenitor population. Unfortunately, early attempts to increase endocrine differentiation and total islet mass by overexpressing *Ngn3 in vivo* have not proved fruitful. In transgenic animals, the expression of *Ngn3* throughout the pancreatic epithelium results in a large increase in the number of glucagon-producing cells, with little to no β cells being formed (Apelqvist et al., 1999; Schwitzgebel et al., 2000). Similarly, the forced expression of *Ngn3* in chick foregut endoderm yields only glucagon^{+} cells (Grapin-Botton et al., 2001). Interestingly, the endocrine phenotype observed after the over-expression of *Ngn3* is identical to that seen in *Hes1*-deficient animals. In the absence of *Hes-1,* which normally represses *Ngn3* transcription in response to Notch signaling, *Ngn3* is over-expressed, thereby leading to an excess of glucagon-producing cells and the depletion of endocrine progenitors (Jensen et al., 2000).

Beta2 was isolated as a transactivator of the insulin gene (see Figure 42.3), but it is actually expressed in all islet endocrine cells types during development and in the adult. It is a direct transcriptional target of Ngn3 (see Figure 42.3). The loss of *Beta2* results in a dramatic decrease in all islet endocrine cell types, which suggests that *Beta2* functions in the expansion of the endocrine population or in endocrine cell survival (Naya et al., 1997). The remaining endocrine cells fail to organize into normal spherical islet structures, which suggests that *Beta2* also functions in islet morphogenesis.

4. Nkx2.2/6.1

Members of the NKX class of homeodomain proteins also have roles in the pancreatic endocrine lineage. Both Nkx2.2 and Nkx6.1 are expressed in most pancreatic epithelial cells during early stages of development; however, by embryonic day 15.5, Nkx2.2 becomes restricted to the endocrine cell population, and Nkx6.1 is found exclusively in insulin-producing cells and scattered cells within the ductal epithelium (Sussel et al., 1998; Sander et al.,

2000). During late gestation, Nkx2.2 can be detected in nearly all hormone$^+$ cells, except for the somatostatin-producing cells. After birth, both genes are found to be expressed in the β-cell population. Mice lacking the *Nkx2.2* gene have no detectable insulin$^+$ cells at any stage that has so far been examined, and they also have a dramatic reduction in the number of glucagon-expressing cells and a more modest reduction in the number of PP$^+$ cells (Sussel et al., 1998). The α and PP cells, although fewer in number, express other known markers of these lineages, which suggests that they are terminally differentiated. The expression of Isl-1 and synaptophysin, which are general markers of islet endocrine cells, is normal in the *Nkx2.2* mutants; this suggests that the loss of *Nkx2.2* does not result in a dramatic loss of endocrine cells in general. Subsequent analysis has revealed that these "extra" endocrine cells in the *Nkx2.2* knockout pancreas were increased numbers of the ghrelin-producing ε-cell population (Prado et al., 2004). Thus, *Nkx2.2* is required for the generation of β cells and for the maintenance and expansion of α and PP cells.

Nkx6.1 gene inactivation results in a highly specific profound loss of second-wave insulin$^+$ cells (after embryonic day 13.5), with no alteration in the numbers of other islet endocrine cell types (Sander et al., 2000). Thus, in the absence of *Nkx6.1,* putative β cells do not adopt an alternate islet endocrine cell fate. Genetic epistasis experiments have demonstrated that *Nkx6.1* functions downstream of *Nkx2.2* in the expansion and terminal differentiation of the β-cell lineage (Sander et al., 2000).

5. GDF11

Growth and differentiation factor 11 (GDF11) is a member of the TGF-β family of secreted growth factors that is expressed in the embryonic pancreas throughout the epithelial component between embryonic days 11.5 and 13.5 (Harmon et al., 2004). During late gestation, GDF11 becomes restricted to acinar cells, and a loss of GDF11 results in a slight decrease in acinar cell mass. This was accompanied by a dramatic increase in the total number of endocrine cells in mutant animals as assessed by the general endocrine marker synaptophysin, which suggests an increased allocation of pancreatic progenitors to the endocrine lineage. Further analysis revealed an increase in the number of Ngn3$^+$ cells earlier during development (embryonic day 11.5) and continuing until late gestation, possibly reflecting both precocious and increased endocrine specification. Similar to the results of the transgenic over-expression of *Ngn3,* increased *Ngn3* expression in GDF11 mutants causes an increase in allocation to the α-cell lineage at the expense of β-cell differentiation.

6. Pax4/6

Two members of the paired class of homeodomain-containing transcription factors have been shown to function in pancreatic endocrine differentiation: Pax4 and Pax6. In the pancreas, Pax4 is specifically expressed in both first- and second-wave insulin-producing cells during embryonic development, and it is maintained in adult β cells (Sosa-Pineda et al., 1997). In the absence of Pax4, β and δ cells fail to differentiate (lacking *Pdx1, Hb9,* and insulin expression), and there are increased numbers of glucagon- and ghrelin-producing cells (Sosa-Pineda et al., 1997; Prado et al., 2004). These data suggest

that either Pax4 is separately required in the β- and δ-cell lineages or that these two islet cell types arise from a common progenitor that is dependent on Pax4. In addition, the increased numbers of α and ε cells suggest that cells that would have become β or δ cells have instead adopted one of these two cell fates or, alternatively, that β and δ cells normally produce something that inhibits the expansion of the α- and ε-cell populations.

In contrast with Pax4, Pax6 is expressed in all endocrine cell types within the pancreas both during embryonic development and in adults; however, the global loss of Pax6 has a specific affect on the α-cell lineage. In the absence of Pax6, there is a complete loss of glucagon-producing cells; the other endocrine cell types are present in reduced numbers, and they fail to organize into normal islet structures (Sander et al., 1997; St-Onge et al., 1997). Mice that lack both Pax4 and Pax6 have a complete loss of all pancreatic endocrine cell types (St-Onge et al., 1997). After birth, Pax6 also functions in the maintenance of the differentiated β-cell phenotype; gene inactivation in the mature endocrine population has no effect on cell number, but it results in diabetes and the decreased expression of some β-cell–specific genes (Ashery-Padan et al., 2004).

7. MafA/MafB

The large Maf proteins are basic leucine zipper transcription factors that were first identified in an avian retrovirus. MafA was identified by several independent groups as an activator of insulin gene transcription (see Figure 42.3; Olbrot et al., 2002; Kajihara et al., 2003; Matsuoka et al., 2003; Kataoka et al., 2004; Zhao et al., 2005). MafA is specifically expressed in second-wave insulin$^+$ cells beginning on embryonic day 13.5 and continuing into adulthood, thus making it a marker of more mature β cells (Matsuoka et al., 2004; Nishimura et al., 2006; Tsuchiya et al., 2006). Despite its indication as a critical β-cell factor, the global inactivation of MafA had no effect on the number of insulin-producing cells during embryonic development. Instead, the loss of MafA causes defects in β-cell gene expression and postnatal β-cell function, thus leading to diabetes (Zhang et al., 2005). The lack of a developmental islet phenotype in the MafA knockout animals may be the result of compensation by another closely related Maf family member that is also expressed in developing endocrine cells, MafB (Artner et al., 2006).

MafB is also capable of activating insulin reporter gene transcription in tissue culture cells, although, in adult islets, it is expressed only in α cells, where it regulates the expression of the glucagon gene (Artner et al., 2006). During embryonic development, MafB is expressed in some Ngn3$^+$ cells and in both first and second wave insulin- and glucagon-producing cells, becoming restricted to α cells soon after birth. Loss of MafB results in a dramatic decrease in mature α and β cells (Artner et al., 2007).

8. Brn4

Brain-4 (Brn4) is a POU-homeodomain–containing protein that is expressed in the developing pancreas, specifically in glucagon-producing cells beginning on embryonic day 10 and continuing into adulthood. However, no defect in α-cell specification or differentiation has been observed in Brn4 mutant animals (Heller et al., 2004). The over-expression of a Brn4 transgene in β cells *in vivo* results in the coexpression of glucagon in insulin$^+$ cells (Hussain et al., 2002).

9. Arx

The Arx homeodomain–containing protein is expressed in scattered cells throughout the pancreatic buds between embryonic days 10.5 and 12.5. The inactivation of *Arx* causes a complete loss of the second wave of glucagon-producing α cells, and this results in severe postnatal hypoglycemia and death, because glucagon stimulates the liver to deposit glucose into the bloodstream during times of fasting (Collombat et al., 2003). The decrease in α cell number is accompanied by an increase in both somatostatin-producing δ cells and β cells. Subsequent analyses strongly suggest that $Arx^{-/-}$ cells are diverted from an α-cell fate toward a β- or/δ-cell fate instead. Indeed, the β-cell transcription factor Pax4 is upregulated in *Arx* mutants, whereas *Arx* is upregulated in Pax4 mutants. Thus, these two genes have opposing actions within the endocrine lineage to establish β or δ cells (*Pax4*) and α cells (*Arx*).

V. PANCREAS/β-CELL REGENERATION AND NEOGENESIS

Diabetes results from an absolute (type 1) or relative (type 2) inadequate functional β-cell mass. Thus, the genes and pathways involved in maintaining or altering β-cell mass are candidates for being affected in diabetic individuals. The functional analysis of these genes may lead to new therapeutic strategies for increasing existing β-cell mass in diabetic patients, and it may facilitate the production of β cells *in vitro* from embryonic or stem cells.

A. Factors That Affect β-Cell Mass

The mass of the β-cell has been shown in animal models to remain stable for the first few weeks after birth and then to gradually increase throughout the life of the organism (Scaglia et al., 1997; Bonner-Weir, 2000a, 2000c), and this is also thought to be true for humans (Butler et al., 2003). The endocrine pancreas undergoes substantial remodeling during the neonatal period, including increased apoptosis and neogenesis with progressive decreases in β-cell replication (Scaglia et al., 1997). The β-cell mass is dynamic, and there is much experimental evidence to show that the β-cell population has the potential to adapt to changing physiologic needs and increased functional demands (Bonner-Weir, 2000a, 2000b). In most situations, β-cell mass increases or decreases in accordance with metabolic demands; for example, β-cell mass increases during pregnancy and with the insulin resistance associated with obesity, whereas it decreases after parturition and after insulinoma implantation (Bernard-Kargar and Ktorza, 2001). Two types of compensation occur after increased demand on the β cells: improved function of individual cells and increased β-cell mass. Functional adaptations include a reduced threshold for glucose-stimulated insulin secretion and increased glucokinase activity, both of which lead to enhanced insulin secretion (Liu et al., 2000). Changes in β-cell mass are achieved by both hyperplasia (an increased number of cells) and hypertrophy (an increased individual cell size).

The adult β-cell population has a slow turnover. At any given time, the number of β cells is determined by the balance of newly forming β cells (via the replication of existing cells and neogenesis from undifferentiated progenitor cells) and β-cell loss through apoptosis. It is estimated that there are 1% to 4% new β cells per day (Finegood et al., 1995). Thus, in the absence of

apoptosis, the β-cell number would double in about a month. Indeed, between months 1 and 3 in the rat, the β-cell number doubles each month. After 3 months, the normal turnover of β cells approaches the replication rate, and, thus, the doubling of β-cell mass does not continue. Although it is generally assumed that pancreatic duct cells retain the ability to generate endocrine cells and form new islets, even late in life, the most recent data available indicate that most if not all new adult β cells arise from preexisting β cells with little to no contribution from stem cells or undifferentiated progenitors (Dor et al., 2004). Regardless of the source of new cells, the endocrine pancreas should be considered a slowly renewing tissue, although the ability of endocrine cells to undergo cell division decreases with age.

In the normal adult pancreas, the presence of local factors such as TGF-β maintain the ducts in a quiescent state (Alvarez and Bass, 1999; Crisera et al., 2000); however, under certain conditions (e.g., pancreatic injury), the proliferation of the common duct is induced, and new lobes of pancreas, including endocrine cells, are formed. Glucose is one of the best stimuli for β-cell replication both *in vitro* and *in vivo* (Swenne and Andersson, 1984; Path et al., 2004). Thus, in a normally functioning pancreas, sustained elevations in blood glucose levels should lead to increased β-cell mass, thereby providing compensation for the increased glucose load. Autopsies of human patients reveal a 40% increase in β-cell mass in obese individuals, which suggests that β-cell compensation does indeed occur with increasing insulin resistance (Butler et al., 2003). Defects in β-cell mass compensation in all probability contribute to type 2 diabetes (Bernard-Kargar and Ktorza, 2001), but trying to identify the complex array of genes that affect this process is likely to be difficult. Any gene product that affects the renewal, proliferation, or turnover of β cells would be a candidate for genes involved in the cause of diabetes.

Several secreted factors and their receptors have been shown to play a role in β-cell mass dynamics. For example, gut hormones such as glucagon-like peptide-1 (GLP-1), gastrin, and cholecystokinin have been shown to be important during β-cell neogenesis, regeneration after injury, and differentiation. The long-acting GLP-1 analog exendin 4 stimulates both β-cell neogenesis from ductal progenitors and the proliferation of existing β cells (De Leon et al., 2003). GLP-1–like compounds are currently being used for the treatment of type 2 diabetes in an attempt to enhance β-cell mass and function (Briones and Bajaj, 2006). In addition, several studies have demonstrated a role for the epidermal-growth-factor–related ligand betacellulin (Huotari et al., 1998) in the differentiation and proliferation of native β cells *in vivo* after partial pancreatectomy and in organ culture (Demeterco et al., 2000; Li et al., 2001, 2004; Huotari et al., 2002). Betacellulin in combination with the TGF-β family member activin A has been shown to convert ductal, acinar, and α-cell lines to insulin-producing β cells capable of secreting glucose in a regulated manner (Mashima et al., 1996; Watada et al., 1996; Ogihara et al., 2003; Ogata et al., 2004). Activin likely plays a role in β-cell specification and/or proliferation *in vivo* as well; defects in activin-receptor signaling during embryogenesis result in islet hypoplasia (Shiozaki et al., 1999; Kim et al., 2000). HGF is a mesenchyme-derived growth factor that stimulates the proliferation of both fetal and adult islets in culture (Otonkoski et al., 1994; Hayek et al., 1995), and, in combination with activin A, it is capable of converting the acinar cell line AR42J into insulin-producing cells (Mashima et al., 1996). It acts on epithelial cells through a membrane-spanning tyrosine

kinase receptor, the product of the protooncogene c-met (Sonnenberg et al., 1993), which is highly expressed in β cells (Watanabe et al., 2003). In transgenic mice, the overexpression of HGF (specifically in insulin-producing cells using the rat insulin II promoter) leads to increased β-cell proliferation, and it protects β cells from apoptosis (Garcia-Ocana et al., 2000). It has also been shown that HGF can enhance islet graft survival and function in both the liver and the kidney (Garcia-Ocana et al., 2003; Lopez-Talavera et al., 2004). The adenoviral delivery of HGF to isolated mouse islets in culture markedly improved β-cell survival and proliferation.

B. Evidence for Pancreas/β-Cell Regeneration

Data regarding the transcription factors involved in the promotion of β-cell proliferation and/or neogenesis are lacking. In several models of pancreas regeneration and ductal metaplasia, the upregulation of Pdx1 is associated with increased ductal proliferation and increased islet neogenesis (Sharma et al., 1999; Song et al., 1999); however, a requirement for *Pdx1* in these processes has not yet been demonstrated. By contrast, the winged helix transcription factor FoxO1 functions normally to inhibit β-cell proliferation and *Pdx1* expression (Kitamura et al., 2002). Thus, the inhibition of *FoxO1* activity in β cells is required for proliferation.

The mammalian pancreas (including that of humans) has significant regenerative potential after insult or injury, although not to the same extent as the liver (Tsiotos et al., 1999; Risbud and Bhonde, 2002). Neogenesis from pancreatic stem cells has been reported to occur in several models of pancreas regeneration, including after β-cell destruction using chemical toxins such as alloxan or streptozotocin (McEvoy and Hegre, 1977; Guz et al., 2001; Risbud and Bhonde, 2002), after the induction of pancreatitis (Gress et al., 1994; Risbud and Bhonde, 2002; Taguchi et al., 2002), after cellophane wrapping (Wang et al., 1995), after partial pancreatectomy (Bonner-Weir et al., 1993; Liu et al., 2000), and after the targeting of inflammatory cytokines to the β cell (Gu and Sarvetnick, 1993).

The replication of preexisting endocrine and exocrine cells is increased three- to fourfold after partial pancreatectomy. Although some of the restoration of β-cell mass is the result of the hypertrophy and hyperplasia of the remaining β cells, most of the new β-cell mass has been proposed to arise from ductal cells in a similar manner to what occurs during pancreas development in the embryo (Bonner-Weir et al., 1993). After partial pancreatectomy, there is a proliferation of ductal epithelium (in which progenitors are thought to reside) and the formation of new ductal complexes. Small ductules differentiate into new pancreatic islets and exocrine tissue, thereby forming new lobes of pancreatic tissue that resemble unoperated pancreata. These studies provide support for the existence of a stem-cell–like population in the adult pancreas (Holland et al., 2004), although the presence of dormant stem cells similar to satellite cells in muscle or of facultative stem cells that are activated in response to certain stimuli in the pancreas has not been proven.

The regeneration seen with partial pancreatectomy seems to recapitulate the pathway of embryonic pancreas development, including increased translation of the critical pancreatic factor Pdx1 in the ductal epithelium after a wave of increased proliferation (Sharma et al., 1999). Ductal proliferation during pancreas regeneration is also accompanied by the increased expression of

the GLUT-2 glucose transporter, which is normally only expressed in fetal ducts and mature β cells (Wang et al., 1995).

C. Genes That Affect β-Cell Proliferation

Progression through the cell cycle requires the activity of the heterodimeric cyclin/cyclin-dependent kinase (CDK) complexes. Progression from G1 phase to S phase is mediated by the D class of cyclins and their partners, CDK4 and CDK6. This particular complex is responsible for the phosphorylation of the retinoblastoma protein, which renders it inactive and frees up transcription factors that allow for cell cycle progression. The β cells seem to be particularly sensitive to the loss of certain cell cycle genes. For example, several recent publications suggest a selective role for CDK4 and cyclin D2 in postnatal β-cell proliferation (Rane et al., 1999; Martin et al., 2003; Mettus and Rane, 2003; Georgia and Bhushan, 2004). CDK4 null mutant mice have a 50% reduction in body and organ size, but they develop surprisingly normally considering the fact that this gene regulates the passage of cells from G1 phase to S phase. The main defects in these mice are infertility as a result of the loss of pituitary lactotrophs and diabetes (when they are $\geq$ 2 months old) as a result of decreased postnatal β-cell proliferation and a gradual loss of β-cell mass with age (Rane et al., 1999; Mettus and Rane, 2003). CDK4 mutants are born with the appropriate number of β cells, which demonstrates that this gene is dispensable for the formation of the endocrine pancreas during embryonic development. Likewise, cyclin D2 mutant animals are born with β-cell mass that is equivalent to that of their control littermates, but they show a decline in β-cell mass beginning when they are 2 weeks old as a result of decreased postnatal β-cell proliferation (Georgia and Bhushan, 2004; Kushner et al., 2005). These mice become diabetic by the time that they are 3 months old. It has recently been shown that the Foxm1 winged helix transcription factor, which regulates a number of cell cycle genes including several cyclins and cdc25B (Leung et al., 2001; Wang et al., 2002; Costa et al., 2003), is also dispensable for embryonic stages of pancreas and islet development, but it is essential for postnatal β-cell replication and the maintenance of β-cell mass (Zhang et al., 2006). Pancreas-specific inactivation of *Foxm1* using a Cre-lox strategy results in diabetes by 9 weeks of age as a result of a gradual loss of β cells postnatally. Taken together, these results lead to the provocative suggestion that *Foxm1* may be involved in tissue regeneration in general and that maintaining *Foxm1* in any cell type would prevent the decline in cell proliferation that occurs with age.

Thus, redundant or parallel pathways likely exist during embryogenesis that ensure the generation of appropriate numbers of β cells, whereas mature β cells are highly susceptible to perturbations in cell cycle gene expression. A possible explanation for this is the fact that, although many cell types express both CDK4 and CDK6, pancreatic β cells express only CDK4 (Martin et al., 2003). The ability to activate these cell cycle genes or to prevent the age-dependent decline in their expression may facilitate the expansion of β-cell mass *in vivo* or *in vitro,* or it may increase the proliferation of β cells in isolated islets before or after transplantation. Indeed, the expression of an activated form of CDK4 in islet β cells using the rat insulin promoter results in β cell hyperplasia and improved insulin secretion without hypoglycemia and without the formation of insulinomas (Hino et al., 2004; Marzo et al.,

2004). Similarly, human islets transduced with a lentivirus expressing activated CDK4 show increased β-cell proliferation (Marzo et al., 2004).

D. Generating Islets/β Cells from Stem or Progenitor Cells: The Challenges

There are several potential sources for the large number of insulin-producing cells that are needed to make "islet transplantation" an option for a greater number of individuals with diabetes (Figure 42.4), including the following: (1) the proliferation and expansion of existing β cells *in vivo* or *in vitro;* (2) the proliferation and expansion of cadaver-derived islets; (3) the induction of β-cell differentiation from endogenous progenitors (embryonic ductal cells) or from adult ductal epithelium; (4) the induction of β-cell differentiation from embryonic stem cells (ESCs); and (5) the transdifferentiation of closely related cell types such as acinar, liver, and intestinal enteroendocrine cells (this concept is not discussed further in this chapter).

All of these avenues are experimentally feasible, and they are currently being examined in animal models (Cirulli et al., 1998, 2000; Bonner-Weir et al., 2000; Cheung et al., 2000; Ramiya et al., 2000; Rooman et al., 2000; Lumelsky et al., 2001; Trivedi et al., 2001; Hori et al., 2002; Blyszczuk et al., 2003; Horb et al., 2003; Kahan et al., 2003; Miyatsuka et al., 2003; Blyszczuk et al., 2004; Cao et al., 2004; Koizumi et al., 2004; Lardon et al., 2004; Nakajima-Nagata et al., 2004; Imai et al., 2005). It may be that more than one of these methods will ultimately be used to derive a steady supply of insulin-producing cells. All of these strategies, with the exception of the directed differentiation of ESCs, require access to or the procurement of particular tissues or cell types that may or may not be sustainable in long-term culture. By contrast, ESCs are known to be stable in culture under the correct conditions, and they can be frozen for long-term storage, with excellent viability after thawing (Hogan et al., 1994). As discussed previously, reliable methods of generating a plentiful supply of islet endocrine cells *in vivo* or *in vitro*

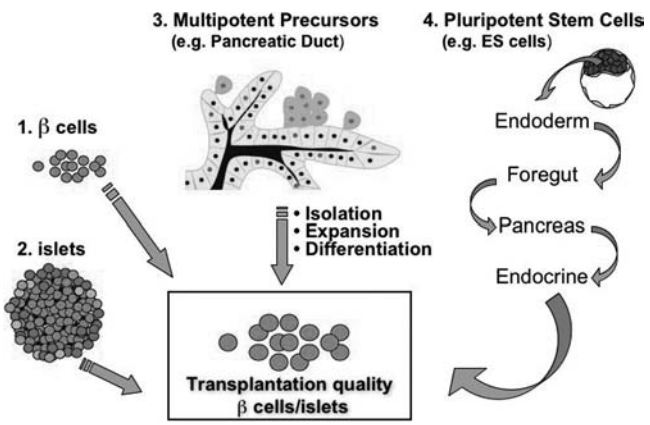

FIGURE 42.4 **Potential sources of transplantable β cells/islets. There are several potential avenues being explored to generate and expand mature β cells or functional islets *in vitro* as a replenishable supply for use in transplantation**: (1) the proliferation and expansion of existing β cells; (2) the proliferation and expansion of cadaver-derived islets; (3) the induction of β-cell differentiation from endogenous progenitors (embryonic ductal cells) or from adult ductal epithelium; and (4) the induction of β-cell differentiation from embryonic stem cells. These strategies are more fully discussed in the text. (See color insert.)

will benefit greatly from the identification and careful manipulation of the factors that promote the proliferation, regeneration, and neogenesis of β cells.

Regardless of the source, experimentally derived insulin-producing cells must be rigorously tested for their survival, their engraftment, their physiologic function *in vivo,* their ability to reverse diabetes and maintain euglycemia, and their lack of tumorigenicity in a relevant animal model. The ability of *in vitro* derived insulin-producing cells to function as mature β cells in an animal model *in vivo* is critical to translating this eventually to human patients. One significant obstacle to the successful transplantation of experimentally derived insulin-producing cells is that current *in vivo* models for evaluating the functionality of such cells are inadequate. Another major limitation of current models is the inability to noninvasively monitor transplanted insulin-producing cells. The desired characteristics of an animal model to evaluate experimentally derived insulin-producing cells would include the following:

- The ability to accept and integrate grafts of human-derived cells or tissues (xenografts)
- The ability to reliably and safely induce diabetes before and after the transplantation of insulin-producing cells
- The ability to alter the expression of factors in the host or graft that may improve graft survival and function
- The ability to monitor β-cell mass noninvasively in clinically relevant sites like the liver
- The ability to retrieve grafted tissue and to assess differentiation and morphologic criteria

E. THE GENERATION OF INSULIN-PRODUCING CELLS FROM ADULT CELL SOURCES

Several models of pancreatic or β-cell injury suggest that the adult ductal epithelium retains some capacity for the production of new β cells (neogenesis). The isolation and culture of ductal epithelium has been shown to yield islet-like clusters that contain functional insulin-producing cells (Bonner-Weir et al., 2000; Ramiya et al., 2000; Trivedi et al., 2001; Ogata et al., 2004). These studies suggest that at least a facultative (if not a genuine) endocrine stem cell exists in the adult pancreatic ducts that, when properly activated, is capable of giving rise to new, functional β cells. In addition, some studies suggest that acinar cells are capable of transdifferentiating directly to insulin-producing cells or of dedifferentiating into a ductal intermediate that can then go on to produce new endocrine cells (Rooman et al., 2000; Lardon et al., 2004; Means et al., 2005). For example, using cultured porcine fetal pancreas, acinar cells were observed to lose exocrine marker gene expression and to dedifferentiate to a multipotent progenitor cell. These immature cells have the capacity to differentiate as insulin-producing cells after transplantation (Humphrey et al., 2001). Islets themselves may also contain stem-like cells that are capable of generating new β cells (Guz et al., 2001; Li et al., 2003), although some researchers claim that the bone marrow harbors organ-specific stem cell populations that are capable of participating in regeneration after injury and possibly of generating new β cells (Ianus et al., 2003). This section will review the evidence for some of the different potential

avenues for deriving therapeutically useful islets or β cells for use in individuals with diabetes.

Because the ductal epithelium is the source of new islet endocrine cells during embryonic development (see Figure 42.1), many investigations have concentrated on trying to generate new islets from fetal and adult ductal tissue. It is not clear whether there exists a quiescent, undifferentiated stem or progenitor cell within the pancreatic ducts and/or islets or whether differentiated duct cells can, with proper stimulation, act as progenitor cells for new acini and endocrine cells (Bouwens, 1998). A recent report suggests that the adult mouse pancreas does contain multipotent precursor cells that represent only ~0.02% of the total cell population, which suggests that a true pancreatic stem cell may indeed exist in the adult (Seaberg et al., 2004). The markers and characteristics of this cell to allow for its identification *in vivo* are not known.

There is also substantial evidence supporting the concept that differentiated duct cells can be activated to produce new endocrine cells in culture. In one study, ducts isolated from adult mouse pancreas were cultured to confluency, at which point islet-producing stem cells budded into the culture medium and formed spherical islet-like structures that expressed and secreted insulin in response to glucose (Cornelius et al., 1997; Ramiya et al., 2000). In addition, these structures rescued an experimentally induced form of diabetes, which suggests that they are functional *in vivo*. These cultures have been maintained for more than 3 years. New ductules that emerge in adult mice after partial pancreatectomy have also been cultured and shown to give rise to small clusters of endocrine cells that contain insulin$^+$ cells (Kim et al., 2004). Similar results have been reported with cultured adult human duct tissue, which suggests that the ductal epithelium that remains after the islet isolation procedure for transplantation may be expanded and directed to differentiate, thus yielding a source of additional islets for transplantation (Bonner-Weir et al., 2000). Several studies have shown that the over-expression of *Ngn3* in duct cultures greatly increases their capacity to give rise to endocrine cells (Heremans et al., 2002; Gasa et al., 2004). Unlike the results from *Ngn3* over-expression *in vivo,* however, these endocrine cells include cells that express insulin.

There is some evidence that genetically marked bone marrow cells can incorporate into pancreatic islets at a low frequency after transplantation into irradiated recipients (Ianus et al., 2003). These cells were reported to activate β-cell–specific genes, including insulin and *Pdx1*. Other studies provide evidence that bone-marrow–derived cells contribute to islet vasculature after injury and not to the formation of new β cells (Mathews et al., 2004). Overall, the ability of bone-marrow–derived cells to act as organ-specific stem cells remains controversial. More work needs to be done to determine whether cells from bone marrow actually differentiate to form cell types from other lineages or whether they provide factors that support the differentiation of new cells from within the host tissue in which they find themselves.

F. THE GENERATION OF INSULIN-PRODUCING CELLS FROM EMBRYONIC STEM CELLS

Several investigators have described methods for the production of insulin-expressing cells from murine ESCs *in vitro* (Lumelsky et al., 2001; Hori et al., 2002; Blyszczuk et al., 2003, 2004; Kahan et al., 2003; Kim et al., 2003; Ku et al., 2004; Miyazaki et al., 2004). Although these protocols are

all similar in that they go through an embryoid body stage in which pluripotent ESCs have already begun to differentiate, they differ with regard to the type of culture medium used, the selection criteria, and the presence or absence of active transgenes to promote β-cell differentiation (reviewed by Kania et al., 2004). In addition, there has been controversy with regard to the validity of the culture methods in which nestin positivity is used as an early selection criteria (Lumelsky et al., 2001; Hori et al., 2002; Miyazaki et al., 2004) and with regard to whether the insulin$^+$ cells observed are actually functional β cells or if they may instead represent an artifact of insulin uptake from the culture medium (Rajagopal et al., 2003; Hansson et al., 2004; Kania et al., 2004; Sipione et al., 2004). As described previously, pancreatic islet cells have many characteristics in common with neurons, both in their gene expression and in their embryonic development. When nestin was identified as a marker of neuronal progenitor cells and selection for nestin$^+$ cells facilitated neuronal differentiation from ESCs in culture (Andressen et al., 2001; Carpenter et al., 2001; Kawaguchi et al., 2001), nestin became a popular candidate as a marker for potential endocrine progenitor cells (Huang and Tang, 2003; Kim et al., 2004). However, more recent studies suggest that nestin expression within the pancreas is restricted to the exocrine lineage (Huang and Tang, 2003; Delacour et al., 2004) or to mesodermally derived cell types such as endothelial cells (Lardon et al., 2002; Selander and Edlund, 2002; Treutelaar et al., 2003).

Thus, although much is known about the factors involved in pancreatic endocrine differentiation during murine embryonic development (reviewed by Jensen, 2004), there is currently a lack of consensus with regard to the best and most reliable method for consistently generating insulin-producing cells from ESCs. Ultimately, however, these strategies must be applied to human ESCs to generate human pancreatic islets or β cells, thereby providing a virtually limitless source of insulin-secreting cells for transplantation therapies. Recently, human ESCs have been shown to be capable of generating neurons (Carpenter et al., 2001) as well as other cell types from all three germ layers (reviewed by Odorico et al., 2001). A recent article reports the directed differentiation of hepatocytes in culture directly from murine ESCs without an embryoid body intermediate (Teratani et al., 2005). These hepatocytes were functional by several criteria, including the rescue of an animal model of liver cirrhosis. Because hepatocytes and pancreatic cells share a common embryologic origin and express many of the same genes, it may be that a similar approach would yield functional β cells in the future.

Several groups have attempted the directed differentiation of murine ESCs into islet endocrine cells using a stepwise approach involving the adding of exogenous factors to the culture medium (Lumelsky et al., 2001; Kim et al., 2003; Ku et al., 2004). Many of the stepwise strategies were not hypothesis driven and were not based on a particular temporal sequence of secreted factors expressed in the pancreas developmentally, because this is in fact not currently known. Thus, in general, these methods have proven difficult to replicate across laboratories. That said, however, some generalities have come from these types of studies, in which insulin-producing cells are generated to a limited extent: (1) all culture strategies go through an embryoid body stage; (2) factors such as FGF, activin, betacellulin, exendin-4, and nicotinamide promote the production of insulin$^+$ cells in the cultures; (3) the percentage of hormone$^+$ cells in the cultures are extremely low; (4) insulin-producing cells

seem to be immature with regard to their gene-expression patterns and their ability to regulate insulin secretion in response to glucose; and (5) many other cell types, including neurons, are also present in the cultures. Even at their best, these methods yield only ~3% insulin$^+$ cells in the entire culture. More recently, Gordon Keller's group has developed the concept that, to generate optimally functional β cells, ESCs must follow the same developmental pathway that endogenous endocrine progenitors follow: definitive endoderm → foregut endoderm → pancreas progenitor → endocrine progenitor → β cell (see Figure 42.4; Kubo et al., 2004; D'Amour et al., 2006).

As mentioned previously, much research has been done regarding the transcription factors involved in pancreas development and islet differentiation, and some of this research is being used to try to force ESCs down a pancreatic/β cell differentiation pathway (Blyszczuk et al., 2003, 2004; Miyazaki et al., 2004). For example, the constitutive expression of either Pax4 or *Pdx1* promotes the development of insulin-producing cells from murine ESCs (Blyszczuk et al., 2003). In Pax4 over-expressing ESCs, *Isl-1, Ngn3,* insulin, islet amyloid polypeptide, and GLUT2 mRNA levels increase significantly. These cells release insulin in response to glucose, and they are able to restore normal blood glucose levels after transplantation into a mouse model of type 1 diabetes.

VI. CONCLUSIONS

The factors that control the specification and differentiation of the different pancreatic lineages continue to be elaborated. A complete understanding of normal pancreas development and endocrine differentiation will undoubtedly facilitate the generation of functional islets *in vitro* for use in the treatment of diabetes. Clearly, it is not sufficient to merely observe insulin reactivity in cultured cells derived from ESCs or any other source and conclude that these cells are β cells. The potential application of these cells to human disease demands a stringency whereby these cells are shown to endogenously produce insulin and to secrete it in a regulatable manner in response to alterations in extracellular glucose concentrations. Although these types of studies can initially be performed *in vitro,* the ultimate test is to determine whether derived insulin-producing cells can fully rescue (i.e., reverse diabetes and maintain glucose homeostasis) an animal whose endogenous β-cell population has been destroyed.

SUMMARY

- The pancreas is composed of two main cell types: exocrine cells and endocrine cells, both of which are derived from the endodermal germ layer.
- It is currently unclear if there exists a multipotent progenitor cell within the embryonic pancreas that is capable of giving rise to both exocrine and endocrine cell lineages.
- Progenitor cells for both the exocrine and endocrine cells are thought to reside within the embryonic pancreatic ductal epithelium.
- Many transcription factors have been identified that are critical for pancreas formation and the differentiation of the endocrine cell populations.

However, less is know about the genes that control exocrine cell fate and differentiation.

- The β-cell mass has the capacity to expand and contract throughout the life of the organism in response to changing metabolic demands.
- In adults, the majority of new insulin-producing β cells come from the proliferation of preexisting β cells, although, under some conditions (e.g., pancreatic injury, obesity), β-cell neogenesis may occur from cells that are located within the ductal epithelium.
- There are currently no good protocols for consistently generating mature, glucose-responsive insulin-producing β cells from adult progenitor cell types or ESCs.
- Attempts to direct the differentiation of facultative or embryonic stem cells toward the β-cell fate will need to rely on a better understanding of the normal developmental processes that take a pluripotent cell from definitive endoderm to foregut endoderm to pancreas progenitor to endocrine progenitor to β cell.

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GLOSSARY

β-cell
The predominant endocrine cell type in the pancreatic islets; it is the only cell in the body that is capable of producing and secreting the hormone insulin.

Diabetes
A heterogeneous group of diseases in which the pancreas fails to produce insulin in sufficient amounts to maintain euglycemia, thus causing a dramatic rise in blood glucose levels.

Endocrine cells
Within the pancreas, the cells that produce hormones such as insulin and glucagon that are secreted directly into the bloodstream, where they travel to their target tissues.

Euglycemia
The state of maintaining blood glucose levels within the normal range (70–120 mg/dL).

Exocrine cells
Within the pancreas, the cells that produce the digestive enzymes that are released into the pancreatic duct and eventually into the duodenum.

Glucose intolerance
An impaired ability to clear glucose efficiently from the bloodstream after a meal; a prediabetic state.

Glucose-stimulated insulin secretion
The increase in insulin released by pancreatic islets is proportional to the elevation in blood glucose; glucose must be metabolized by the β-cell for insulin to be released.

Insulin resistance
The physiologic state in which insulin target tissues in the periphery (mainly liver and muscle) become insensitive to insulin, thereby requiring elevated plasma insulin levels to elicit the same biologic effect.

Islets
Spherical clusters of pancreatic endocrine cells that are comprised mainly of insulin-producing β cells.

Islet transplantation
The experimental surgical procedure being used to treat some patients with type 1 diabetes; cadaver donor islets are transplanted into the liver of patients via the portal vein, where they then begin to secrete insulin and reverse diabetes.

Mature onset diabetes of the young
A dominant monogenic form of type 2 diabetes.

Neogenesis
The process of generating new β cells from stem or progenitor cells.

Neurogenin 3
A basic helix–loop–helix transcription factor that is essential for the generation of all pancreatic islet cell types.

Partial pancreatectomy
A surgical procedure used in experimental animal models to stimulate pancreas and β-cell regeneration; a portion of the pancreas is removed, and new pancreas tissue is generated from the remnant organ.

Type 1 diabetes
An autoimmune disease in which the insulin-producing β cells are specifically destroyed.

Type 2 diabetes
A disease that is usually associated with obesity in which the β cells fail to produce enough insulin to overcome peripheral insulin resistance.

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43

EARLY LIVER DEVELOPMENT AND HEPATIC PROGENITOR CELLS

JAY D. KORMISH and KENNETH S. ZARET

Cell and Developmental Biology Program, Fox Chase Cancer Center, Philadelphia, PA

INTRODUCTION

The liver has many functions in health and disease, and thus there is much interest in the mechanisms of liver development and regeneration. The large size of the liver and its relative simplicity in terms of resident cell types make it an attractive experimental model for revealing general principles of development and progenitor cell biology. There are only two primary cell-type decisions that are made in the generation of the primary functional cell types of the liver. The first occurs when the embryonic endoderm generates the nascent liver cell, a hepatoblast, as opposed to other tissue progenitors, and the second occurs when the hepatoblast generates hepatocytes and cholangiocytes (bile duct cells). During development, the liver is a site of hematopoiesis, which is an essential function for the embryo. Thus, early liver growth is rapid, and vascular development is tightly integrated into hepatic morphogenesis. The rapid growth of the embryonic liver and its accessibility by relatively simple dissection techniques has enabled tissue explant and biochemical studies that have been unfeasible for other developing organ systems. Consequently, our understanding of liver organogenesis includes the genes and signals that induce cell-type decisions and mechanistic insights regarding how chromatin is regulated by transcription factors to execute the hepatic program. This chapter will focus on the experimental approaches and findings that have arisen from studies of early liver development in embryos and regenerating livers in adults and how they have provided insights that can be applied to stem cell differentiation.

The adult liver secretes many serum proteins; they help control serum osmotic pressure and are carriers of lipids and other molecules. The adult liver also controls metabolite levels in the bloodstream and detoxifies ingested compounds that are taken up by the intestine and transferred to the liver via the hepatic portal vein. Acute toxicant ingestion can lead to the destruction of hepatocytes, but the cells have remarkable regenerative powers. Chronic liver

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damage, whether elicited by toxicants, genetic disease, or viral infection, can lead to states in which mature hepatocyte proliferation is impaired and resident progenitor cells are activated. Because the liver is essential for viability and livers suitable for transplantation are in short supply, there is intense interest in generating hepatocytes from progenitor cells in the liver as well as from exogenous sources (e.g., transdifferentiation from other tissues or *de novo* differentiation from embryonic stem cells [ESCs]). In addition, being able to generate hepatocytes at will *in vitro* would greatly facilitate drug development, e.g., by allowing prospective compounds to be tested for their inhibitory activities, or their ability to elicit toxic molecular profiles. Furthermore, if hepatocytes could be readily generated from different human genetic backgrounds, it would allow drug leads to be prescreened for patient-specific sensitivities. An understanding of the factors required for liver development and progenitor cell biology will enhance the ability to generate hepatocytes for these purposes.

I. TWO ENDODERMAL ORIGINS OF EMBRYONIC LIVER CELLS

The embryonic liver morphologically emerges from a single budding region of the developing ventral foregut. By contrast, the pancreas emerges from separate dorsal and ventral regions of the gut, and, later, the dorsal and ventral buds fuse to create the gland (Slack, 1995; see Chapter 42). However, when early time points of mouse foregut development were recently studied, the hepatoblasts that compose the liver bud were discovered to emanate from two spatially distinct populations of endoderm cells that are brought together, during gut closure, to create a single liver bud (Tremblay and Zaret, 2005; Figure 43.1). This finding arose from a fate-mapping study of

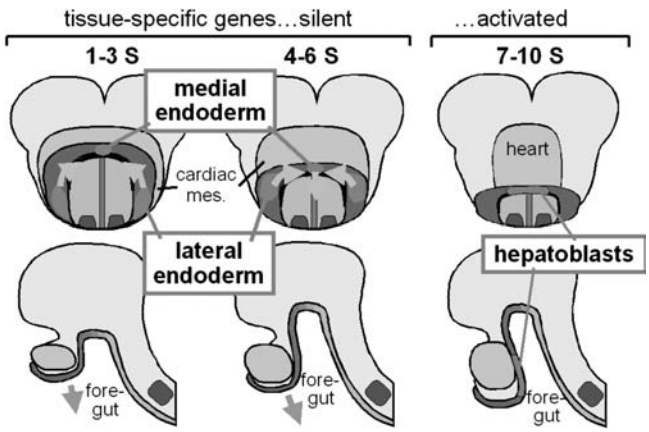

FIGURE 43.1 **Distinct endoderm domains contribute to the liver bud. Upper panels,** Frontal views of the anterior portion of mouse embryos, looking into the foregut. **Lower panels,** Parasagittal views. The figure depicts medial and lateral endoderm domains *(pink)* at different stages that contribute cells to the liver bud as determined by fate-mapping studies described in the text. The 1–3 and 4–6 somite pair stages precede liver specification. By the 7–8 somite pair stage, the prospective hepatic endoderm has converged at the ventral midline, and liver-specific gene expression commences. The green arrows depict the direction of the movement of cells. In the upper panels, the cells move ventrally and toward the midline. In the lower panels, the movement of cells enlarges the foregut. (See color insert.)

the foregut endoderm in which a lipophilic fluorescent dye was injected into patches of endoderm cells in different parts of the mouse embryonic foregut, before foregut tissue specification. The time of dye injection, which took place on embryonic day 8.0 (2–6 somite pairs), is the time at which the endoderm resides on the exterior, bottom surface of the embryo. This is during the beginning of foregut invagination, before gut tube closure. Embryos were cultured whole, in their yolk sac, until the organogenic phase of foregut gut development (embryonic day 9.0–9.5) and the tissue bud(s) in which the dye-injected cells resided were documented.

The fate map results revealed paired lateral domains of endoderm cells on the left and right sides of the embryos, which, when labeled, primarily gave rise to liver bud cells (see Figure 43.1). The fate mapping also revealed a small domain of cells at the ventral midline that, when labeled, left a trail of descendants in the midline of various ventral foregut tissues and terminated within the rostral portion of the liver bud. This apparently multipotent medial progenitor population in the mouse appears to correspond with a similarly migrating population of cells seen in chick embryos (Kirby et al., 2003). In summary, liver bud cells arise from two functionally and spatially distinct progenitor domains (lateral and medial) that are morphogenetically brought together during foregut closure and that give rise to the liver bud.

It is presently unknown whether common or distinct signals specify the liver in the medial and lateral hepatic progenitor domains. In this context, it is interesting to note that dorsal and ventral pancreas bud specification involves differences in signaling mechanisms (Deutsch et al., 2001; Kumar and Melton, 2003) and regulatory transcription factors (Ahlgren et al., 1997; Li et al., 1999; Bort et al., 2004). If there are differences in specifying the medial and lateral liver progenitors, it may indicate that there will be different ways to generate hepatocytes from nonliver progenitors and stem cells. Furthermore, genetic lineage studies will be necessary to determine whether adult descendants of the medial and lateral liver progenitors exhibit differences in hepatocyte function, including regenerative capacity. Considering that the discovery of different natural progenitors of the liver bud was made only recently, the rest of this chapter will consider the endoderm as a single embryonic source of liver progenitor cells.

II. DEVELOPMENTAL COMPETENCE OF THE VENTRAL FOREGUT ENDODERM, THE SPECIFICATION OF HEPATOBLASTS, AND THE EMERGENCE OF THE LIVER BUD

A. Competence of the Endoderm to Initiate Hepatogenesis: Role of FOXA and GATA Transcription Factors

The specification of the liver from the foregut endoderm has been defined as the time in development when the transcription of liver-specific genes initiates in endoderm cells and when it can be maintained by the cells outside of the normal embryonic context. During the foregut development of the mouse, the *albumin* gene is among the earliest to be expressed in the liver (Gualdi et al., 1996). *Albumin* expression is limited to the endoderm of the foregut region at the 7- to 8-somite stage (approximately embryonic day 8.5), and it remains active in foregut endoderm tissue explants (Cascio and Zaret, 1991; Gualdi et al., 1996). Although the midgut endoderm normally does not

express *albumin,* isolating it from the mesoderm and ectoderm and culturing it *in vitro* causes the ectopic expression of *albumin* (Bossard and Zaret, 2000). Conversely, the *in vitro* association of midgut mesoderm inhibits *albumin* induction in foregut endoderm explants (Gualdi et al., 1996). However, by embryonic day 13.5, the midgut-associated endoderm has lost the capacity to activate *albumin* when removed from its associated mesoderm. These observations show that both the foregut and the midgut endoderm are competent to express the liver-specific gene before embryonic day E13.5, but they can be inhibited from doing so by mesodermal interactions. Recent studies in frogs and mice suggest that inhibition of Wnt signaling in the foregut but not the midgut is responsible for the observed difference in competence between the foregut and midgut (A. Zorn, personal communication). The expression of Wnt signaling inhibitors in the foregut but not the midgut during early endoderm development strengthens this model (Finley et al., 2003).

Studies of factors controlling the expression of the *albumin* gene have revealed an explanation for developmental competence. The *albumin* gene contains an upstream enhancer element that is sufficient for liver-specific expression in transgenic mice (Pinkert et al., 1987). A variety of liver-enriched transcription factors have been found to bind and regulate the *albumin* enhancer in adult liver cells (DiPersio et al., 1991; Liu et al., 1991; Jackson et al., 1993; Bossard and Zaret, 1998; 2000). Interestingly, *in vivo* footprinting studies on E11.5 midgut, which does not express albumin but is competent to do so, showed that solely the FOXA and GATA binding sites of the enhancer are occupied in endoderm before *albumin* induction (Bossard and Zaret, 2000). *Foxa1/Hnf3α, Foxa2/Hnf3β, Foxa3/Hnf3γ, Gata4,* and *Gata6* transcription factor genes are expressed during the formation of the definitive endoderm, and they continue to be expressed in endodermal organs, including the liver (Ang et al., 1993; Monaghan et al., 1993; Sasaki and Hogan, 1993; Morrisey et al., 1998). Strikingly, the FOXA and GATA sites of the *albumin* enhancer become unoccupied on embryonic day 13.5, which is when the midgut endoderm loses its competence to express *albumin* (Bossard and Zaret, 2000). These observations suggest that FOXA and GATA factors play a key role in the endoderm's competence to respond to developmental cues on embryonic day 13.5.

Biochemical studies on *in vitro* reconstituted chromatin templates have shown that FOXA1 and, to a lesser extent, GATA4 can bind to target DNA sites in compacted chromatin and expose an underlying nucleosome (Cirillo et al., 2002). This has led to a model that stating that, in the endoderm, FOXA and GATA proteins act as "pioneer factors" by being among the first to bind to regulatory elements of genes during development and by helping to make the chromatin competent to be expressed in an endodermal organ (Zaret, 2002).

Revealing the role of *Foxa* and *Gata* genes during liver development has been complicated by their functional redundancy and by their roles in gastrulation and the development of extraembryonic endoderm, before liver formation. The postgastrulation lethality associated with the *Foxa2* homozygous mutant (Ang and Rossant, 1994; Weinstein et al., 1994) was initially overcome with the use of chimeric embryo technology (Dufort et al., 1998). In these experiments, *Foxa2* homozygous null ESCs that were genetically marked with *lacZ* were injected into wild-type blastocysts, which were then implanted into a foster mother. The failure of *Foxa2*$^{-/-}$ cells to

survive in the foregut and midgut endoderm of the chimeric embryos demonstrated that *Foxa2* is intrinsically necessary in those tissues (Dufort et al., 1998). Tissue-specific gene inactivation using the cre/lox system has also allowed for a bypass of early embryonic lethality. Here, the endogenous gene of interest is flanked by loxP sequences. In the presence of cre recombinase, which is driven by a tissue-specific promoter, the lox sites recombine and result in the excision of the intervening DNA. This technology has been used to inactivate *Foxa2* function in the context of a *Foxa1* deletion, the latter of which, by itself, is not lethal to embryos (Kaestner et al., 1999). Inactivation of both *Foxa1* and *Foxa2* in foregut endoderm demonstrated that the factors are redundantly required there for liver specification (Lee et al., 2005), thus supporting the chromatin competence model.

GATA transcription factors have been found to play a similar critical role during early endoderm and liver development in the frog, fish, chicken, and mouse models of development (Stainier, 2002). In zebrafish, the morpholino knockdown of *Gata4* has shown that it is necessary for liver specification (Holtzinger and Evans, 2005). Similar experiments with *Gata6* have shown that it is not required for specification, but that it is required for the subsequent expansion of the fish liver bud (Holtzinger and Evans, 2005). *Gata4* and *Gata6* have been detected in the developing foregut and liver bud in the mouse (Bossard and Zaret, 1998; Morrisey et al., 1998; Jacobsen et al., 2002). Like those of the forkhead factors, the studies of *Gata4* and *Gata6* genes in mammalian development have been complicated by the early embryonic lethality associated with the mutants (Kuo et al., 1997; Morrisey et al., 1998). Chimeric embryo experiments showed that *Gata4* is required in endoderm for foregut development, but a later role in liver development has not been investigated (Narita et al., 1997). The aggregation of *Gata6* mutant ESCs and tetraploid wild-type ESCs allows a bypass of the yolk sac function of *Gata* factors, and this has demonstrated that *Gata6* is required for liver bud expansion (Zhao et al., 2005). *Gata4* may compensate for the early loss of *Gata6* expression and allow for the liver specification that is observed in the *Gata6* mutant. Such a prediction could be tested with the double inactivation of *Gata4* and *Gata6* during liver bud specification. In zebrafish and chicken, *Gata5* is expressed in the developing foregut endoderm and liver (Laverriere et al., 1994; Reiter et al., 2001). Through the use of the morpholino-based knockdown of *Gata5* in zebrafish, *GATA5* has been found to be necessary for endoderm and liver development (Reiter et al., 2001). A similar function of *Gata5* in mammalian foregut and liver development is unlikely, because *Gata5* appears to not be expressed in the developing gut (Morrisey et al., 1998)

B. Signaling Control of Liver Progenitor Cell Fate

Embryo tissue transplant and explant systems in the chick and mouse have been fundamental for revealing signaling events that lead to liver specification. From such studies in the chick, it was initially determined that interactions with the cardiac mesoderm are necessary for the specification of the liver (Le Douarin, 1975; Houssaint, 1980; Fukuda-Taira, 1981; Gualdi et al., 1996). Further studies with the mouse determined that fibroblast growth factors (FGFs) produced by the cardiac mesoderm were essential for this induction (Gualdi et al., 1996; Jung et al., 1999, Calmont et al., 2006).

In later experiments, it became apparent that tightly associated septum transversum mesenchyme cells were present in ventral foregut explants and that they were a source of bone morphogenetic protein (BMP) ligands (Rossi et al., 2001). BMPs alone could not induce liver gene expression, but they were found to be necessary in conjunction with FGFs for the endoderm to initiate hepatogenesis. Similar conclusions have been obtained with studies in the chick (Zhang et al., 2002; 2004). Interestingly, knowledge of the liver-inducing properties of BMP and FGF in embryos has been successfully applied to *in vitro* protocols for differentiating embryonic stem (ES) cell to liver-like cells (see Section V below).

In subsequent studies of mouse FGF signaling during liver specification, it was determined that, in the absence of FGFs, the ventral foregut endoderm initiates the expression of genes for the ventral pancreas (Deutsch et al., 2001). The ventral pancreas is located immediately caudal to the liver domain, and it arises from the portion of the ventral foregut that normally has little or no contact with the cardiac endoderm. This has led to a model which states that the ventral foregut is bipotential for liver or pancreas fate, depending on contact with the cardiac mesoderm and the extent of exposure to FGF (Deutsch et al., 2001; Bort et al., 2004). This model has been extended by the observation that higher levels of FGF signaling are required for the specification of the lung just rostral to the liver region (Serls et al., 2005). The specification of the lung, liver, and ventral pancreas thus appears to be determined, at least in part, by the endoderm's duration of association with the cardiac mesoderm and with different consequent levels of FGF signaling.

In zebrafish, Wnt signaling has been found to be necessary for liver specification. An elegant forward genetic screen was performed to identify defects in endoderm organ development during zebrafish development (Ober et al., 2006). The mutant *prometheus* was identified and found to encode the Wnt2bb ligand. By various means, the authors showed that *prometheus* and β-catenin signaling is required for the early development of the zebrafish liver. Interestingly, even though the entire liver bud is missing cells during early development, a liver can still form a bit later than normal. It is believed that cells from the adjacent endoderm, possibly the gall bladder, can be recruited to initiate the formation of a liver bud in *prometheus* mutants, and once recruited possess the remarkable regenerative capacity to restore the developing liver. By this model, the Wnt2bb ligand would not be necessary for the specification of the later-appearing liver cells.

Other signaling pathways have been studied with regard to their roles in the specification of foregut organs. In the zebrafish, retinoic acid is required for liver and pancreas specification (Stafford and Prince, 2002). In this system, it appears that retinoic acid acts as a morphogen to determine the caudal–rostral placement of the liver and pancreas near the end of gastrulation. When retinoic acid signaling is inhibited, the liver and pancreas are not specified. When retinoic acid signaling is enhanced by the addition of ectopic retinoic acid, the pancreas is specified at a more anterior position. Interestingly, intrinsic Hox gene expression does not seem to control foregut endoderm tissue specification; rather, as described previously, signaling from adjacent mesoderm has a primary role in cell-type specification. Furthermore, there is little evidence that a Hox code controls genes in the mesoderm that, in turn, specifically govern foregut patterning.

C. Concomitant Morphologic Transitions to the Liver Bud: Hex, Endothelial Cell Signaling, and Growth Zones

After specification, there appear to be three stages of liver bud morphogenesis during which the nascent hepatoblasts transform from a simple epithelial layer into a mass of emergent liver bud cells (Bort et al., 2006). During the first phase, the nascent hepatoblast cells, which are contiguous with the endodermal epithelium, elongate away from the apical/luminal surface. This causes the epithelium to thicken and the cells to take on a columnar appearance. During the second phase, the nuclei of the hepatoblasts appear stratified as the result of the basal-to-apical migration of the nuclei progressing through cell division. During the third phase, the basal lamina degrades, and the hepatoblasts delaminate from the epithelium and migrate into the surrounding mesenchyme, creating a liver bud.

Studies of the homeodomain gene *Hex* have shown that this transcription factor gene is critical for the second stage of hepatic bud development (Bort et al., 2006). In *Hex* null homozygotes, hepatoblast nuclei fail to undergo migration, and the cells consequently fail to delaminate. Later, *Hex* mutant hepatoblasts fail to maintain liver gene expression and eventually appear to take on a duodenal cell fate. The knockdown of *hHex* in zebrafish, using a morpholino approach, has also shown that the gene is required for liver development after hepatic specification (Wallace et al., 2001). The liver bud expression pattern of *hHex* in developing chick and frog embryos suggests that the gene plays similar roles in these organisms (Newman et al., 1997; Yatskievych et al., 1999). A second transcription factor gene, *Prox1,* appears to be required during the third phase of liver development, described above (Sosa-Pineda et al., 2000). In the *Prox1* mutants, hepatic cells fail to delaminate and form a proper liver bud. This defect appears to result from defects in the normal sequence of E-cadherin downregulation and basal lamina breakdown.

After the initial phases of liver bud outgrowth, the liver bud undergoes the extensive proliferation and migration of hepatic cells into the septum transversum mesenchyme (STM). The transcription factors H2.0-like homeobox gene (Hlx) and LIM-homeobox gene-2 (Lhx2) and the signaling molecules BMP4, hepatocyte growth factor (HGF), and transforming growth factor β are expressed by the STM, and they are implicated in hepatoblast proliferation (Schmidt et al., 1995; Hentsch et al., 1996; Amicone et al., 1997; Porter et al., 1997; Rossi et al., 2001; Weinstein et al., 2001; Kolterud et al., 2004; Wandzioch et al., 2004). Hlx and Lhx2 presumably promote the expression of the signaling factors expressed by the STM.

In addition to the STM, endothelial cell precursors are recruited to or induced within the liver bud area, and these cells also promote liver bud migration and proliferation. A mouse mutant that is defective in endothelial cell differentiation, *flk1,* exhibits a failure in liver bud outgrowth (Matsumoto et al., 2001). The stimulatory effect of endothelial cells in wild-type liver bud explants, apart from the circulatory system, showed that the relevant factor(s) are produced locally by the endothelium. In adult livers, the release of HGF seems to be a paracrine factor released by endothelial cells to promote hepatocyte proliferation (LeCouter et al., 2003). The identification of additional signaling molecules involved in this endothelial–hepatocyte interaction may reveal factors that are crucial for liver regeneration.

Studies in chicken liver development have shed light on how the liver organ expands and undergoes further differentiation (Suksaweang et al., 2004). It appears that, in each liver lobe, differentiation from a hepatoblast to a functioning hepatocyte starts at the center of the lobe and expands to the periphery of the organs. The periphery of each lobe is associated with highly proliferative growth zones. The expanding growth zones appear to contain cells which are associated with mesenchymal cells. The alteration of the expression of β-catenin showed that the inhibition of Wnt signaling is required for the maintenance of the hepatic growth zones and further differentiation (Suksaweang et al., 2004).

III. HEPATOBLAST DIFFERENTIATION INTO HEPATOCYTES AND CHOLANGIOCYTES (BILIARY CELLS)

A. Roles of HNF6, HNF-1β, and HNF4 Transcription Factors

In the mouse, *HNF-1β* is expressed in the tubular structures of the liver, and it has been found to play an intrinsic role in biliary development (Coffinier et al., 2002). A cre recombinase driven by an *alb/afp* promoter and a floxed allele of endogenous *HNF-1β* gene has been used to specifically reduce the expression *HNF-1β* in the developing liver. Livers that are deficient for *HNF-1β* have large, malformed bile ducts and a failure of smaller bile ducts to differentiate. In these livers, there is also a concomitant loss of interlobal arteries. Because *HNF-1β* is not normally expressed in the interlobal arteries, it is thought that their defect is caused by the bile duct defect. This phenotype emphasizes the interplay between bile duct cells and endothelial cells during liver development. In addition to obvious defects in bile duct morphology, several genes associated with bile duct function (e.g., bile acid synthesis enzymes, fatty acid oxidation enzymes) are reduced in expression in the *HNF-1β* mutant. The role of *HNF-1β* in biliary development may be evolutionarily conserved in vertebrates, because the knockdown of *HNF-1β* in zebrafish shows a similar defect in biliary tree elaboration and bile secretion defects (Matthews et al., 2004). Further studies in zebrafish suggest that *HNF-1β* could play an even earlier role in liver development during foregut patterning. *HNF-1β* is expressed in the developing zebrafish foregut and later in the liver, pancreas, and hindgut (Sun and Hopkins, 2001; Gong et al., 2004). The morpholino knockdown of *HNF-1β* function during foregut development has shown that *HNF-1β* is absolutely necessary for pancreas development and that it influences liver specification (Sun and Hopkins, 2001).

HNF6/OC1, which is a member of the onecut family, has been shown to play an essential role in liver development. Studies in the zebrafish have determined that *HNF6* is necessary for proper biliary tract development (Matthews et al., 2004). In zebrafish, *HNF6* is expressed in the developing liver and pancreas and later only in the liver, gall bladder, and proximal intestine. In the morpholino knockdown of *HNF6*, there is a defect in biliary tree development and bile secretion. Both mammalian and zebrafish studies have determined that *HNF6* controls the expression of many other important transcription factors. *HNF-1β* appears to be a prime transcriptional target for *HNF6*, because in the *HNF6* knockdown, *HNF-1β* transcription is reduced (Matthews et al., 2004). Indeed, *HNF6* and *HNF-1β* morpholino knockdowns result in identical

phenotypes. This has led to the model that suggests that the phenotype observed in the *HNF6* mutant is primarily the result of a defect in *HNF-1β* expression.

B. Signaling Control: Role of Notch Signaling and Alagille Syndrome

In both the zebrafish and the mouse, there is growing evidence that the Notch signaling pathway plays a significant role in the decision between hepatocyte and biliary cell fates. Hints for this role came from the study of Alagille syndrome in humans, which is characterized by defects in tubular structures throughout the body, including in the bile duct system in the liver. Human patients with Alagille syndrome frequently have mutations in the Notch receptor *jagged1* (Li et al., 1997; Oda et al., 1997); this led to more in-depth studies of the Notch signaling pathway in mice. Mice that are double haploinsufficient for the *jagged1* ligand and the *Notch2* receptor or that are homozygous for a hypomorphic *Notch2* allele display defects in bile duct differentiation (McCright et al., 2002). In these mutants, fewer cells form a bile duct precursor, and those that do form precursors never form a mature duct. A morpholino approach has been used to knock down the function of several members of the Notch pathway during zebrafish embryo development (Lorent et al., 2004). The *Notch2/5* double knockdown results in a complete loss of the bile duct lineage and an increase in the hepatocyte lineage. The *jagged 2/3* double knockdown results in a similar (although less penetrant) phenotype. In the reciprocal experiments, ectopic Notch expression results in ectopic biliary ducts. Taken together, the mouse and zebrafish studies show that the Notch pathway is required for the proper formation and differentiation of the bile duct cell lineage from a hepatoblast. In the mouse (McCright et al., 2002), *jagged1* is expressed in the hepatic portal vein and arteries, and *Notch2* is expressed in the surrounding hepatocytes. Thus, the notch pathway is likely activated in hepatoblasts that are in close proximity of the liver endothelial cells that express several *jagged*-type Notch receptors.

C. Maturation of the Hepatocyte: Evidence for Complex Genetic Networks

Initial evidence for liver transcription factor networks came from genetic studies. Using mouse genetics, *HNF6* was found to be required for the stable expression of *Foxa2* and *HNF4* (Landry et al., 1997). In zebrafish morpholino knockdown studies, *HNF6* is required for *HNF-1β* expression and for wild-type levels of *HNF4* and *Foxa2* expression (Matthews et al., 2004). In the mouse, HNF4, which is a nuclear receptor–type transcription factor, has been found to be required for liver differentiation after bud formation (Li et al., 2000; Parviz et al., 2003). A proximal-promoter region sufficient for high levels of liver expression of *HNF4* has been identified. A combination of footprinting, DNA mobility shifting, and chromatin immunoprecipitation techniques have revealed binding sites for HNF-1α, HNF-1β, Sp-1, GATA6, HNF6, and COUP-TFII in the *HNF4* promoter (Hatzis and Talianidis, 2001). In liver-derived cell lines, high levels of *HNF4* expression require synergism between HNF-1α and HNF6 or HNF1β and GATA6. These experiments and others involving chromatin immunoprecipitation (Kyrmizi et al., 2006) have led to a model depicting the complex interregulatory relationships among the transcription factors, i.e., a network that drives hepatic differentiation.

The above genetic and promoter studies have been vastly expanded by the recent advent of ChIP-on-chip studies. In this technique, DNA fragments that are isolated via chromatin immunoprecipitation are amplified and hybridized to microarrays displaying a tiled representation of the genome. The key advantage of this technique is that many transcription factor binding targets can be identified in one experiment. This has allowed for the expansion of known factor binding sites and the construction of regulatory networks of transcription factors required for liver development (Odom et al., 2004). In experiments performed by Odom et al. (2004), chromatin was isolated from human hepatocytes and pancreatic islets and immunoprecipitated with antibodies against HNF-1α, HNF4α, and HNF6. When liver and pancreatic targets were compared, HNF-1α, HNF6, and HNF4 were found to be bound to distinct sets of genes and a significant subset of common genes, apparently reflecting the close developmental relationship of the two tissues. HNF-1α and HNF4 consistently occupied the same promoters, and many genes were co-occupied by all three transcription factors. HNF6 was suggested to support a feed-forward loop in which HNF6 binds the *HNF4* promoter and HNF4, in turn, binds many genes that are bound by both factors.

The cross-regulatory relationship of the key transcription factors required for liver development has been expanded by additional ChIP-on-chip studies of HNF-1α, HNF4, FOXA2, HNF6, CREB1, and USF1 in liver chromatin (Odom et al., 2006). In all cases except USF1, the factors were found to bind their own promoters. In many cases, cross regulation was evident. For example, HNF4 and HNF-1α are bound to each other's promoters, *Foxa2* is bound by HNF6, and HNF6 and HNF-1α are bound to the *HNF4* promoter. Furthermore, HNF-1α, HNF4, FOXA2, and HNF6 occupy the promoter of the *transthyretin* gene, which is a commonly studied liver gene. These results have further validated the combinatorial model for tissue-specific gene expression, and they indicate that the transcription factors that control multiple genes during development are likely to display autoregulation. This autoregulatory feedback is hypothesized to impart stability to the expression of key transcription factors driving organ differentiation.

Interestingly, the auto- and cross-regulatory relationships of endodermal transcription factors, including FOXA1 and FOXA2, appear to have been fixed at the onset of metazoan evolution (Davidson and Erwin, 2006). From a comparison of the transcription factor networks that govern endoderm development, it was hypothesized that, within all metazoans, there exists an essentially immutable "kernel" network for endoderm development. This fundamental kernel provides the basis for "add-on" regulatory programs that differ among different metazoans. It remains to be determined to what extent the regulatory relationships of liver transcription factors unveiled by Odom et al. (2006) represent a fixed network for liver among other metazoans.

IV. ADULT LIVER STEM CELLS

The mature liver is composed of functional units called *lobules* (Figure 43.2; Saxena et al., 1999; Fausto and Campbell, 2003). Lobules are approximately hexagonal-shaped structures that are composed of epithelial sheets or "plates" of hepatocytes. The hepatocyte plates are 1 to 2 cells thick, and they radiate from the central vein located in the center of the lobule to portal tracts at

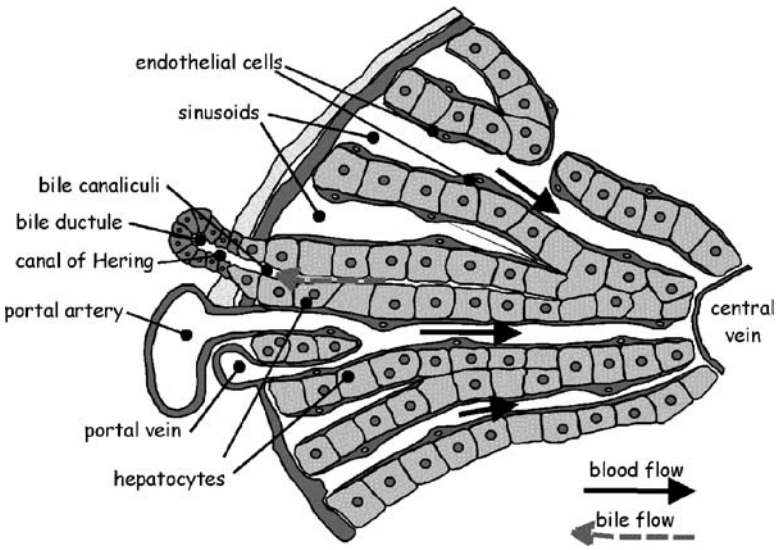

FIGURE 43.2 **Cross section of the liver lobule.** Hepatocytes are organized into sheets of cells that radiate from the central vein to the portal tract. The portal tract (triad) is composed of the bile duct, the portal vein, and the portal artery. Blood flows from the portal tract through the sinusoidal spaces and to the central vein. Bile is secreted from the apical surface of the hepatocytes into the bile canaliculi. The canals of Hering are composed of cells that connect the bile ducts with the bile canaliculi. It is the cells of the canals of Hering that proliferate to generate oval cells during chronic liver damage. (Figure adapted from Bloom and Fawcett, 1994.)

the periphery. The portal tracts contain branches of the portal vein that carry blood from the intestine and the hepatic artery. Blood flows in an outward-in direction, from portal to central, through the sinusoidal spaces between the hepatic plates. Opposite from the sinusoidal/basal surface of the hepatocyte epithelium, bile is secreted into apical spaces called *bile canaliculi*. The bile canaliculi connect to intrahepatic bile ducts, which are composed of cholangiocytes in the portal tracts and which further connect to drain bile into the gall bladder.

After acute liver damage (e.g., partial hepatectomy), hepatocytes rapidly replicate to reconstitute the liver mass (Rhim et al., 1994; Overturf et al., 1997; Laconi et al., 1998; Fausto and Campbell, 2003). On the basis of the high regenerative capacity of the liver and plate-like formation of hepatocytes within the lobule, it was originally thought that hepatocytes were generated from a stem cell niche located close the central vein, with descendants that stream distally to the portal tracts as they mature. However, this has proven to not be the case (Ng and Iannaccone, 1992; Bralet et al., 1994). Instead, it appears that virtually all hepatocytes have the capacity to replicate during normal growth and during acute liver damage (Rhim et al., 1994; Overturf et al., 1997). Bile duct cell replication appears to occur in response to an increase in biliary pressure, such as what occurs during biliary obstruction or damage (Slott et al., 1990). Biliary duct cell replication is evident in all parts of the tubular duct system, and the collective effect is to elongate the system. Like hepatocyte regeneration in response to acute damage, there has been no evidence for bile duct cell expansion via a stem cell niche (Slott et al., 1990).

In contrast with the situation with acute liver damage, recent studies have identified stem cell–like compartments in the adult liver that are activated in response to chronic liver damage (Thorgeirsson, 1996; Sell, 2001; Fausto and Campbell, 2003; Knight et al., 2005). Various chemical genetic models and human disease models have been used to mimic chronic liver damage, in which hepatocyte replication is compromised and a progenitor or stem cell population is activated (Shinozuka et al., 1978; Sell and Salman, 1984; Evarts et al., 1987; Germain et al., 1988; Dabeva and Shafritz, 1993; Factor et al., 1994; Sigal et al., 1995; Overturf et al., 1996). Careful work has shown the hepatic precursor cells/oval cells as originating from the canals of Hering, which is the ductular region that connects the hepatic canalicular system to the biliary tree (Paku et al., 2001). As the oval cells proliferate, they form tubular structures that maintain contact with the terminal bile ductual and distal hepatocytes. This structure is thought to maintain the flow of blood and bile while regeneration occurs. Furthermore, oval cell proliferation seems to be distinct from cholangiocyte proliferation, which occurs after bile duct obstruction (Slott et al., 1990; Paku et al., 2001). Oval cells are bipotential and have the capacity to form both hepatocytes and cholangiocytes (Pack et al., 1993; Nagy et al., 1994; Yin et al., 2002; Qin et al., 2004). However, proliferating biliary cells can only form other biliary cells (Slott et al., 1990). At this point, it is important to note that oval cells are likely not to be actual liver stem cells but are the fast replicating daughters of such a hypothesized cell type. The position and character of the true liver stem cell is still to be determined.

Several laboratories have observed the stem cell–like properties of oval cells when they are transplanted into host livers (Yasui et al., 1997; Wang et al., 2003a). An example of the *in vivo* stem cell property of the oval cell came from experiments done by Wang et al. (2003a) with a mouse model. In these experiments, oval cell proliferation was induced with the carcinogen 3,5-diethoxycarbonyl-1,4-dihydrocollidine, and oval cells were isolated by size via fractionation and cell sorting on the basis of cell surface markers. The isolated cells were transplanted into mice that were genetically deficient for fumarylacetoacetate hydrolase (*Fah;* Overturf et al., 1996). *Fah*-deficient mice develop chronic liver damage, which in turn induces oval cell proliferation. Adding different levels of 2-(2-nitro-4-trifluoro-methyl-benzyolyl)-1,3-cyclohexanedione (NTBC) reduces liver toxicity and prolongs the time over which liver regeneration must occur. When *Fah*-positive donor cells are introduced into a *Fah*-negative host, NTBC withdrawal imposes a selection for the proliferation of *Fah*-positive donor cells. The *Fah* mutant and wild-type alleles serve as genetic markers to distinguish between the host and donor cells, along with mismatched sex donors and hosts. When *Fah*-positive oval cells were introduced into a *Fah*-negative host, the donated cells were found to repopulate the liver. When differentially marked mature hepatocytes were coinjected with isolated oval cells, both oval cells and hepatocytes were able to equally contribute to liver regeneration. Overall, these results confirm the repopulating capacity of the oval cell in the compromised liver. Cell culture experiments demonstrated that oval cells can differentiate into both the hepatocyte and the bile duct cell lineages (Pack et al., 1993; Nagy et al., 1994; Yin et al., 2002; Qin et al., 2004). Future studies are needed to rigorously determine the capacity of oval cells to contribute to both hepatocytes and bile duct cells in the *in vivo* model.

The origin of oval cells has also been a subject of recent debate. Are oval cells a remnant of early embryonic hepatoblasts, are they generated later in development, or do they originate from non-liver sources? The morphologic changes of the hepatoblasts during development and the repopulating capacity of fetal hepatic cells have shed light on the potential developmental origins of oval cells. α-Fetoprotein (AFP) is expressed at high levels only during fetal development of the liver. The re-expression of AFP and other fetal markers during oval cell proliferation suggests that these cells may have a less mature hepatic character (Sell et al., 1974; Hayner et al., 1984; Sell and Salman, 1984; Lemire and Fausto, 1991; Alpini et al., 1992). Furthermore, during liver development, hepatoblasts near endothelial structures express markers that are indicative of both hepatocytes and bile duct cells. A similar expression of two differentiation markers is also seen in oval cells during their expansion after chronic liver injury (Yaswen et al., 1984; Germain et al., 1985). Like oval cells, hepatoblasts can be cultured and induced to form cells of both hepatocyte and cholangiocyte character (Rogler, 1997; Kamiya et al., 1999; Strick-Marchand and Weiss, 2002). Also, freshly isolated hepatoblasts and hepatoblasts that have been cultured can be reintroduced into the liver and repopulate both lineages of liver cells, under normal and chronic injury conditions (Dabeva et al., 2000; Sandhu et al., 2001; Malhi et al., 2002; Suzuki et al., 2002; Minguet et al., 2003; Strick-Marchand et al., 2004). Careful genetic lineage tracing during development will be needed to further refine the hepatoblast–oval cell lineage model.

Oval cell/hepatic precursor cells (Omori et al., 1997; Petersen et al., 1998; Theise et al., 1999; Crosby et al., 2001; Petersen et al., 2003) and fetal hepatoblasts (Suzuki et al., 2002; Lazaro et al., 2003; Nava et al., 2005) express certain markers in common with hematopoietic stem cells (HSCs). *In vitro* experiments have demonstrated that a subset of bone marrow stem cells express AFP and c-kit (an HGF receptor) and that these stem cells can be induced to express hepatocyte and bile duct markers when they are exposed to appropriate stimuli (Oh et al., 2000; Miyazaki et al., 2002; Schwartz et al., 2002; Lee et al., 2004). However, *in vivo* experiments are not able to effectively recapitulate this *in vitro* transition. Well-controlled transplant experiments demonstrate that HSC conversion into hepatocytes is rare to negligible (Petersen et al., 1999; Alison et al., 2000; Fogt et al., 2002; Wagers et al., 2002; Ng et al., 2003; Menthena et al., 2004). Recently, the rare HSC transdifferentiation events observed in chronic injury models have been attributed to the fusion of HSCs to hepatocytes (Vassilopoulos et al., 2003; Wang et al., 2003b). The variable ploidy of hepatocytes and the ability of macrophages to fuse with other cells make this a plausible model (Vassilopoulos et al., 2003). Attempts to refute the fusion model have been made, but more sensitive fusion detection systems need to be employed to make this argument more convincing (Harris et al., 2004). Therefore, it appears that the majority of oval cells arise from the liver and that they express many markers in common with other stem cell tissues (Omori et al., 1997; Petersen et al., 1998; Theise et al., 1999; Crosby et al., 2001; Petersen et al., 2003).

A poorly defined subset of cells begins to proliferate in the periductual structures of the liver lobule under conditions that stimulate oval cell production (Sell, 2001). These cells do not appear to form functional bile duct cells, and they have been speculated to be mesenchymal in origin, perhaps stellate cells (mobile mesenchymal cells of the mature liver) or infiltrating hematopoietic stem cells

(Paku et al., 2001). Although the origin and purpose of these cells remain unclear, the vicinity to proliferating oval cells suggests that they may be part of a niche that either stabilizes oval cells or that activates them during chronic damage. Given the important role of the STM and endothelial cells during embryonic hepatoblast proliferation and migration (Schmidt et al., 1995; Amicone et al., 1997; Matsumoto et al., 2001; Rossi et al., 2001; LeCouter et al., 2003; Wandzioch et al., 2004), the mesenchymal origin of the periductal cells near oval cells may not be surprising, and it suggests that signaling events resembling those in development could be involved.

V. DIFFERENTIATION OF LIVER-LIKE CELLS FROM EMBRYONIC STEM CELLS

The difficultly in obtaining and isolating human oval cells and fetal hepatoblasts has led to the search for other sources of cells that could be used for liver regeneration via stem cell transplants. Mouse ESCs derived from the cells of the early blastocyst embryo can be cultured, manipulated genetically, and reintroduced into the developing blastocyst, where they can reconstitute all tissues of the developing embryo (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998; Reubinoff et al., 2000). Several groups have been able to promote the differentiation of ESCs into hepatocyte-like cells both *in vitro* and *in vivo*, albeit not very efficiently (Chinzei et al., 2002; Yamamoto et al., 2003; Teratani et al., 2005; Yamamoto et al., 2005). The differentiation of ESCs toward an hepatoblast phenotype reduces their frequency of teratoma formation (Chinzei et al., 2002). The partial differentiation of ESCs into hepatocyte precursors may allow for an expansion and enrichment of cells that will effectively contribute to liver tissue.

A major hurdle in defining the final differentiation of hepatocytes has been the ability to distinguish hepatoblasts from visceral endoderm. Visceral endoderm is formed early during embryo development, and it later forms the extraembryonic tissue of the yolk sac. The visceral endoderm and yolk sac function together in a role that is similar to that of the liver before liver development (Meehan et al., 1984; Sellem et al., 1984; Duncan et al., 1997), and thus the yolk sac expresses many of the same genes that are diagnostic of liver differentiation (Meehan et al., 1984; Abe et al., 1996). More careful experiments have found genes that are differentially expressed in the liver and the yolk sac, and these markers have been used to confirm the formation of hepatocyte-like cells from ESC lines (Jones et al., 2002; Asahina et al., 2004; Kubo et al., 2004; Stamp et al., 2005; Tada et al., 2005).

Signaling molecules that are known to influence endodermal cells during embryo development, such as activin, are being used to promote ESCs to differentiate along a mesendodermal fate (Schuldiner et al., 2000; Kubo et al., 2004; D'Amour et al., 2005; Tada et al., 2005). *Brachyury, Foxa2*, and *goosecoid* have been used as markers for this transition (Blum et al., 1992; Showell et al., 2004), along with cell sorting to enrich for definitive endoderm cells. Cells expressing markers of liver, lung, and intestine differentiated from such cells, although again at low efficiency (Kubo et al., 2004; Tada et al., 2005). Mimicking what has been found in embryology, many studies have found that FGFs promote the differentiation of ESC to liver-like cells

in vitro (Hamazaki et al., 2001; Kuai et al., 2003; Yamamoto et al., 2003, 2005; Teratani et al., 2005). Notably, Gouon-Evans et al. (2006) recently added BMPs (Rossi et al., 2001) to the differentiation protocol and more consistently and robustly generated hepatocyte-like cells from mouse ESC. Interestingly, they also saw co-differentiation of endothelial cells at the periphery of their hepatocyte-like cultures; as in development (Matsumoto et al., 2001), conditions that enhanced endothelial yield, enhanced hepatic cell yield. It thus seems likely that the further enhancement of hepatocyte differentiation from ESCs will be facilitated by the additional application of principles that apply to normal liver development.

SUMMARY

- The liver is specified from two functionally and spatially distinct domains of ventral foregut endoderm. FGF, BMP, and Wnt signaling appear to be required for liver specification.
- Hepatoblasts proliferate and migrate away from the ventral foregut. Intrinsic factors as well as signals from the septum transversum mesenchyme and endothelial-derived mesenchyme are required for liver bud development.
- After acute damage, hepatocytes undergo massive replication to reconstitute the damaged liver. Only during chronic liver damage, when hepatocyte replication is compromised, does a bipotential hepatic precursor cell/oval cell population begin to proliferate to repair the liver.
- Oval cells originate near the canals of Hering. Nonliver cell types, such as HSCs, do not appear to significantly contribute to liver cell repopulation.
- Presently, the most successful approaches to generating hepatic precursor cells from ESCs employ the signaling events and intrinsic transcription factors that are required for normal liver development.

GLOSSARY

Canal of Hering
A small population of cells that connect the bile ducts to hepatocytes. These cells allow for the flow of bile from the bile canaculi to the bile ducts.

Cholangiocyte
The cell type that comprises the bile duct system.

Commitment
The point in development when a specified tissue can no longer respond to development signals and becomes set in its gene expression profile.

Competence
The point in development at which a field of cells becomes competent to respond to signaling cues for cell-type specification.

Hepatoblast
The bipotential embryonic liver precursor cell for the adult hepatocyte or cholangiocyte.

Hepatocytes
The functional metabolic cells of the mature liver. These cells form polarized sheets or "plates" in the liver. The basal surface is in contact with endothelial cells, which allows for the transfer of metabolites to and from the blood stream, whereas the apical surface secretes bile into canaliculi, which form between adjacent hepatocytes.

Oval cells
A subset of cells that originate from the canals of Hering and that proliferate to reconstitute both hepatocytes and cholangiocytes during chronic liver damage. Oval cells are hypothesized to either constitute or to be related to the bipotential hepatic precursor cell population (hepatoblasts).

Specification
The point in development at which a field of cells, such as the one in the ventral foregut, starts to express markers that are specific to an organ and at which the expression of such markers is stable when the endoderm is cultivated outside of the embryo.

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INTESTINAL STEM CELLS IN PHYSIOLOGIC REGENERATION AND DISEASE

DAVID H. SCOVILLE,[1,3] XI C. HE,[1] GOO LEE,[2] TOSHIRO SATO,[1] TERRENCE A. BARRETT,[2] and LINHENG LI[1,3]

[1]*Stowers Institute for Medical Research, Kansas City, MO*

[2]*Northwestern University Feinberg School of Medicine, Department of Medicine and Microbiology/Immunology, Division of Gastroenterology, Evanston, IL*

[3]*University of Kansas School of Medicine, Department of Pathology and Laboratory Medicine, Lawrence, KS*

INTRODUCTION

After normal development, many tissues maintain the ability to regenerate for repairing damaged tissues and lost cells by emulating earlier developmental pathways. The intestine represents an ideal system for the study of adult tissue stem cells because of its well-organized structure and its need for constant cellular regeneration. Intestinal stem cells, which are maintained in a less-differentiated state, are required for both the initial development and maintenance of the crypt–villus structure. In this chapter, we discuss the signaling pathways responsible for controlling stem cell behavior and epithelial cell differentiation. Finally, we discuss how alterations in these pathways contribute to cancer development in the intestine.

I. OVERVIEW OF INTESTINAL ANATOMY AND FUNCTIONAL HISTOLOGY

The intestinal tract is composed of an endodermally derived epithelium and a mesodermally derived stroma. It differentiates from the primitive gut tube into several different regions with varying functions (see Chapter 40). beginning rostrally, the gastrointestinal tract is composed of the nasopharynx, the esophagus, and the stomach, which perform bulk transport and mechanical and enzymatic digestion. next, the small intestine is divided along its length into the duodenum, the jejunum, and the ileum, which aid with both digestion

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and absorption. the duodenum absorbs electrolytes, monosaccharides, and water- and fat-soluble vitamins; the jejunum absorbs protein and fat; the ileum is responsible for vitamin b12 and bile salt uptake. finally, the colon concentrates the remaining fecal contents. for the purposes of this chapter, our discussion will be limited to the small intestine and the colon.

The small intestinal architecture is composed of contiguous villi and crypts. The villi, which are lined by a single layer of columnar epithelium, extend into the intestinal lumen and contain terminally differentiated cells; alternatively, the crypt, which harbors the proliferative potential of this tissue, is the result of epithelial invaginations into the gut mucosa. Intestinal stem cells (ISCs), which possess the capacity for self-renewal and the generation of multipotent transient amplifying progenitors, reside near the crypt base (Booth and Potten, 2000). Progressing upward from the intestinal stem cell position, the transient amplifying progenitors occupy the rest of the crypt structure, whereas functionally mature cells are located along the villus (Hermiston and Gordon, 1995). Thus, the crypt–villus architecture can be divided into proliferation and differentiation compartments (Figure 44.1).

Mature intestinal epithelial cells are derived from transient amplifying progenitors that specify absorptive and secretory lineages. The absorptive lineage contains only one mature cell type, enterocytes, whereas the secretory lineage includes three differentiated cell types: goblet cells, enteroendocrine cells, and Paneth cells. Enterocytes, goblet cells, and enteroendocrine cells migrate upward from the crypt to the villus, eventually undergoing apoptosis at the villus tip and shedding into the lumen, a process that takes about 3 to 5 days in mice. Interestingly, Paneth cells, which differentiate from secretory progenitor cells within the crypt, do not follow the migrational pattern of the other cell types. Instead, they migrate in the opposite direction toward the crypt base. In addition, Paneth cells also have a longer life span.

Enterocytes, which are located along the villus, are the most abundant cell type in the small intestine, and their primary function is to absorb nutrients from the intestinal lumen. To aid in this process, these cells secrete hydrolases

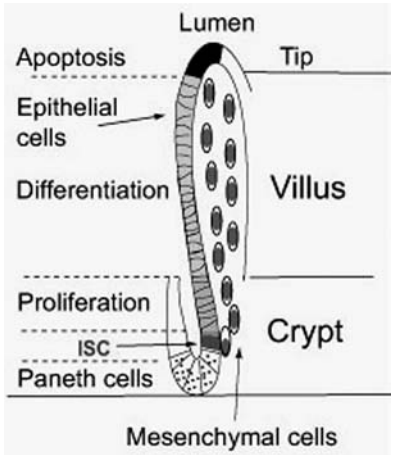

FIGURE 44.1 **Intestinal structure and compartmentalization.** The small intestine is composed of crypts and villi which correspond to compartments of proliferation and differentiation, respectively. Crypts contain ISCs and progenitors, as well as differentiated Paneth cells at the base. The other three mature cell types are located along the villus.

and contain an enzyme-rich, brush-like border along the apical microvilli that serves to dramatically increase the surface area available for digestion and absorption. These enzymes break down small peptides and carbohydrates into components that can be easily absorbed. Goblet cells function to secrete mucins that protect the epithelium from the harsh digestive environment of the intestinal lumen. These cells—and, correspondingly, the mucus content in the gut—increase in number from the duodenum to the colon. Enteroendocrine cells comprise less than 1% of the total population of intestinal epithelial cells, making them the rarest differentiated cell type. Different types of enteroendocrine cells exist, and they are classified on the basis of the molecules that they secrete, such as serotonin, cholecystokinin, substance P, and secretin (Evans and Potten, 1988; Hocker and Wiedenmann, 1998). These hormones and neuropeptides are secreted from the basolateral membrane, and they act in an endocrine fashion, in part to regulate gastrointestinal motility. Paneth cells function to control microbial growth within the gastrointestinal tract by secreting several antimicrobial agents, including cryptdin, defensins, and lysozyme (Ayabe et al., 2000; Porter et al., 2002). These secretions can also be used in immunohistochemical staining to distinguish this cell type.

The structure of the colon is slightly different in that it lacks villi as well as Paneth cells. The stem cells are proposed to reside at the bottom of the crypts; progenitor cells occupy the bottom two thirds of the crypt up from the stem cell region, whereas that one third of the crypt closest to the lumen is occupied by terminally differentiated cells (Booth and Potten, 2000). In other words, the structure and division of cellular compartments in the colon are the same as that seen in the small intestine, with the exception that there are neither Paneth cells nor villi present.

II. INTESTINAL STEM CELLS

Although direct functional proof of ISCs has yet to be demonstrated, significant evidence supports their existence. Historically, the presence and location of the putative ISCs have been recognized using clonal analysis techniques, DNA-labeling studies, and immunohistochemistry. Initially, the monoclonal nature of intestinal crypts was established using various chimeric or heterozygous mutant mice strains. It was observed that individual crypts were completely composed of cells either from only one of the mouse strains, such as in the case of chimeras (Hermiston et al., 1993; Roth et al., 1991; Schmidt et al., 1988), or they exhibited the same mutant gene expression profile, such as in the case of heterozygous mice (Bjerknes and Cheng, 1999; Roth et al., 1991). Furthermore, experiments in which the intestinal epithelium was subjected to a cytotoxic agent showed that a single surviving cell could regenerate the entire crypt (Potten, 1995). These results can be explained by a common stem cell yielding a monoclonal crypt cell population of multilineage progenitors, whereas villi, which are maintained by several crypts, are as a result polyclonal (Booth and Potten, 2000). Furthermore, DNA-label–retaining experiments using bromodeoxyuridine (BrdU) or ^{3}H-thymidine both supported the existence of ISCs and localized them to a position near the crypt base (Potten et al., 2002). Experimentally, these labels incorporate into DNA and diminish with each cell division; however, cells that retain the label over long periods of time are thought to be predominantly slow cycling or in the quiescent state. Although

progenitors are rapidly proliferating, adult tissue stem cells are thought to be slow cycling, and thus they can be distinguished using this method. Alternatively, long-term label retention can be explained by DNA segregation in asymmetric stem cell division. Recent evidence indicates that stem cell chromatids are preferentially segregated such that one chromatid is always retained in the parent stem cell, thereby yielding an "immortal chromatid" (Cairns, 1975). This could imply that label retention by these specific crypt cells identifies a characteristic stem cell function. Although mechanistic details remain unknown, this unique segregation would ensure that ISC DNA is preferentially protected from mutagenesis, thus decreasing the risk of carcinogenesis in this highly proliferative epithelium (Potten et al., 2002; Shinin et al., 2006).

Immunohistochemical evidence has shown that long-term BrdU-retaining cells do not co-stain with lysozyme (a specific Paneth cell marker) or Ki67 (a cell proliferation marker), thus distinguishing these cells and designating them as putative slow-cycling ISCs (He et al., 2004). So far, much research has focused on finding novel and specific ISC markers in an attempt to further pinpoint their location. Molecules such as Musashi-1, phosphorylated phosphatase homologue of tensin (P-PTEN), phosphorylated Akt (P-Akt), and 14–3-3ζ have been found to be expressed in long-term BrdU retaining cells in the proposed ISC position as well as in some progenitor cells (Batlle et al., 2002; Booth and Potten, 2000; He et al., 2004; Korinek et al., 1998; Potten et al., 2003). These studies and others have been useful for localizing the ISC to a position about four to five cells above the crypt base (Potten, 1995). Alternatively, other experiments identified candidate stem cells intermingled with Paneth cells. These experiments showed that the chemical treatment of mice could mutate the lectin-expression profile of a single morphologically columnar cell. These cells, which are located at the crypt base, were found to give rise to mutant clones containing multiple cell types. In addition to their mixed cell population, the generation time for these clones indicated a stem cell or long-term progenitor origin (Bjerknes and Cheng, 1999). Despite these correlative experiments, demonstrative proof of putative stem cells using an appropriate *in vivo* method for functional characterization remains elusive. This is in part the result of technical challenges in isolating pure ISCs. However, recent reports have indicated the possibility of isolating fractions significantly enriched for ISCs using flow cytometry (Dekaney et al., 2005).

Putative ISC markers have also provided insights into stem cell regulatory pathways. Potential interactions between the epithelium and adjacent stromal tissue have been an active area of research in an attempt to define the intestinal stem cell niche. Studies regarding signals emanating from the niche have and will provide insight into our understanding of stem cell self-renewal and proliferation as well as cell fate determination.

III. STEM CELL/NICHE INTERACTIONS

The idea of a stem cell niche, which was conceived with regard for hematopoietic stem cells, proposed that a specific microenvironment exists for these unique cells (Schofield, 1978). This concept of a stem cell niche has carried over into the study of other adult stem cell systems. From pioneering experiments in Drosophila, *Caenorhabditis elegans,* and other mammalian systems, some basic and overarching concepts regarding stem cell niche function have been revealed (Doetsch, 2003; Fuchs et al., 2004; Li and Xie, 2005; Lin, 2002;

Spradling et al., 2001). The stem cell niche is composed of a special group of cells that provide a microenvironment to which stem cells directly attach. Although other mesenchymal microenvironments may exist along the length of the crypt–villus axis, niche interactions serve to specifically inhibit the differentiation and control the self-renewal and proliferation of stem cells. Because cellular regeneration is initiated at the stem cell level, regulating the properties of these cells is critical. Myoepithelial fibroblasts in the crypt epithelium are proposed to be a niche cell candidate in part as a result of both their proximity to and expression of prospective stem cell regulatory molecules (He et al., 2004; Mills and Gordon, 2001). Although incomplete, several stem cell–niche interactions have been well defined. In intestinal development, Hedgehog signaling from epithelial cells to mesenchymal cells serves to inhibit ectopic crypt formation within the villi and areas adjacent to already forming crypts. Thus, during development, Hedgehog signals ensure appropriate crypt spacing and numbers (Madison et al., 2005). Recently, Hedgehog signaling has also been shown to play a role in controlling proliferative signaling in adult intestinal tissue by restricting Wnt target gene expression in the colon (van den Brink et al., 2004). Although Wnt signaling is well known to promote small intestinal epithelial propagation, recent studies demonstrated that signaling through bone morphogenic proteins (BMPs) represents a mechanism for suppressing ISC proliferation (Haramis et al., 2004; He et al., 2004). In addition, various signals, including Wnt and Notch, are involved in cell fate determination. Unraveling these complex signaling mechanisms has led to a better understanding of the molecular and physiologic interactions that control stem cell fate as well as potential causes of tumorigenesis within the intestine (Radtke and Clevers, 2005).

IV. SIGNALING MECHANISMS CONTROLLING INTESTINAL STEM CELL SELF-RENEWAL, PROLIFERATION, AND DIFFERENTIATION

A. Wnt Signaling

Wnt signaling is known to play several roles within the intestine. First, it maintains stem cells via cell cycle control (Sancho et al., 2004). Second, Wnt signals mediate cell fate determination. Third, Wnt signaling controls the localization of cells along the crypt–villus axis.

Although a variety of different types of Wnt signaling can occur, canonical Wnt pathway activation controls β-catenin localization. When the Wnt signal is not present, the complex of adenomatous polyposis coli/glycogen synthase kinase 3 beta (APC/GSK3β) can bind β-catenin (Munemitsu et al., 1995; Rubinfeld et al., 1996; Rubinfeld et al., 1993; Su et al., 1993). GSK3β, a protein kinase, can then phosphorylate β-catenin, thereby targeting this molecule for proteosomal destruction (Giles et al., 2003). Without the Wnt signal, T-cell factors (TCFs) act as transcriptional repressors. Conversely, Wnt receptor activation results in the nuclear localization of β-catenin, where it binds members of the TCF transcription factor family, activating target gene expression (Figure 44.2; Giles et al., 2003; Nusse et al., 1997; Peifer and Polakis, 2000; Sahl and Clever, 1994a, 1994b). Thus, upon ligand binding, Wnt/β-catenin activation in the ISC converts TCF into a transcriptional activator that initiates stem cell activation via the upregulation of genes such as cyclinD and c-myc, which are well known for promoting cell cycle progression.

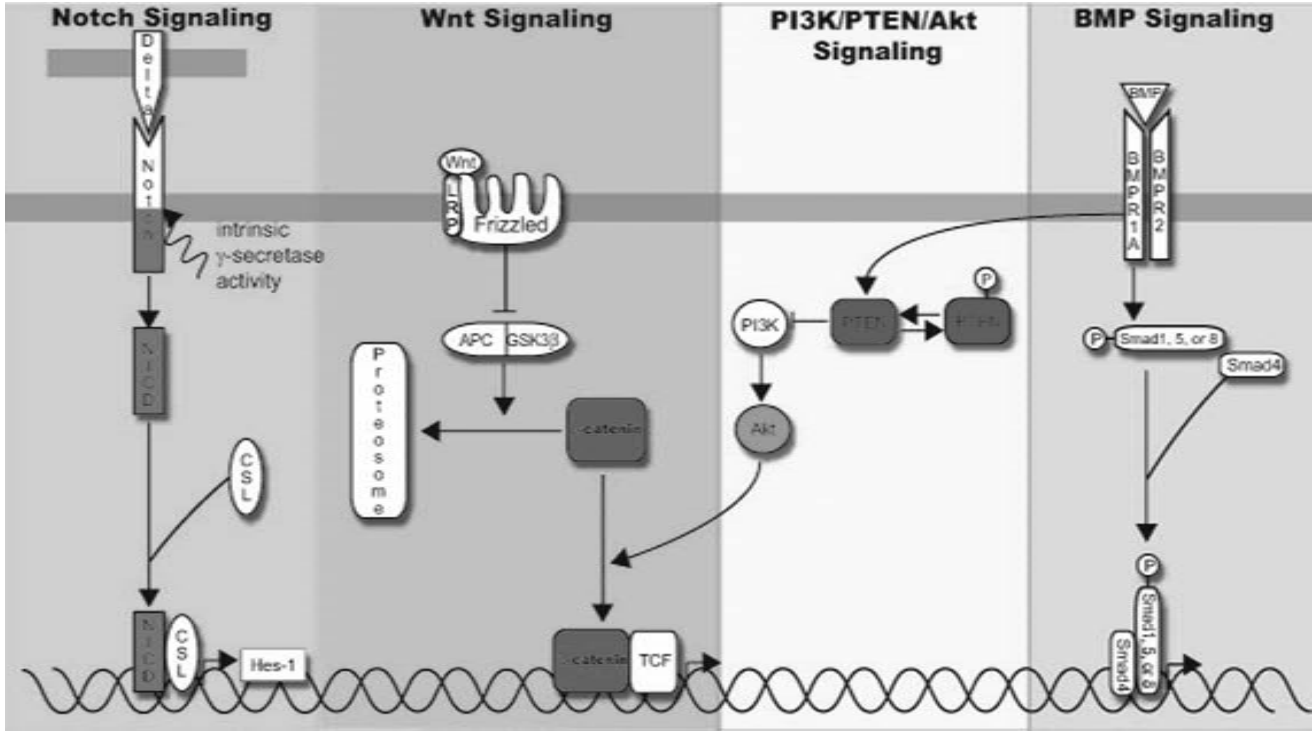

FIGURE 44.2 **ISC signaling pathways.** Wnt, Notch, BMP, and PTEN and PI3K pathways are involved in maintaining ISCs and differentiation programming within the intestine. Notch signaling results in transcriptional activation which drives differentiation of absorptive progenitors and is also a proliferative signal within the crypt. Wnt signaling results in nuclear localization of b-catenin which when bound to TCF factors converts them from repressors to transcriptional activators upregulating genes involved in activating the ISC cell cycle. PI3K signaling activates Akt which can assist Wnt in regulating b-catenin in certain types of cells including ISCs. PTEN controls Akt activation by inhibiting PI3K. Although the exact mechanism remains unknown, BMP signaling can inhibit proliferation in ISCs perhaps in part through the Smad transcription factors and in part via interaction with PTEN.

Normally, stem cells undergo niche-dependent asymmetric division. This division results in a daughter cell, which migrates outside the niche, and a stem cell, which remains attached to the niche. Upon the completion of cell division, niche interaction ensures the return of TCF repression in the stem cell, whereas the more differentiated daughter cell progresses through the proliferation and differentiation program as a result of continued nuclear β-catenin/TCF-driven gene expression (Pinto et al., 2003; van de Wetering et al., 2002). Experimental evidence supports this model, because the deletion of TCF4 results in an absence of proliferating cells and a loss of crypt formation (Korinek et al., 1998).

Additionally, APC has been shown to control crypt epithelial proliferation. In humans and mice, hereditary mutation of one of the APC-encoding alleles creates a predisposition for numerous intestinal adenomas and subsequent carcinoma development in familial adenomatous polyposis (FAP). This APC mutation allows for the constitutive nuclear localization of β-catenin, which results in overactive target gene expression and an enlargement in crypt size as a result of an increase in proliferating cells (Groden et al., 1991; Joslyn et al., 1991; Korinek et al., 1997; Miyoshi et al., 1992; Nakamura et al., 1991). In fact, mutations in the Wnt signaling cascade, including APC, may be the initiating event in most human colorectal cancers (Bienz and Clevers, 2000).

Wnt signaling also affects cell fate determination by promoting general secretory lineage commitment as well as by driving Paneth cell gene expression (Figure 44.3). Evidence for this is based on several mouse intestinal

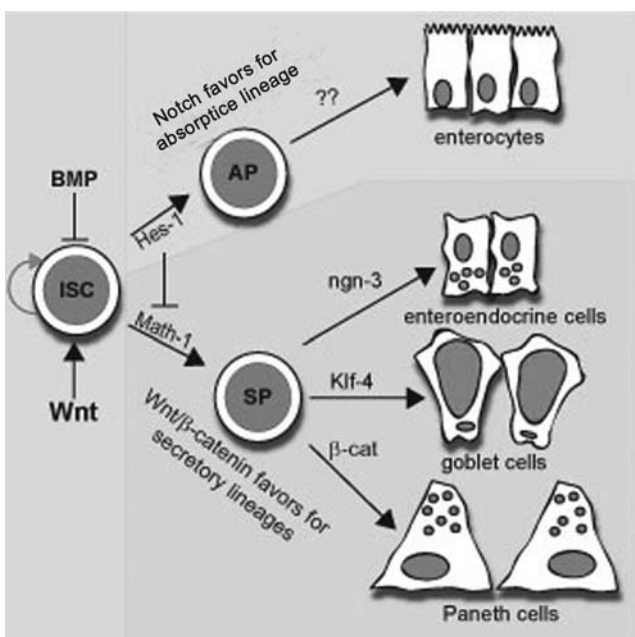

FIGURE 44.3 **Intestinal cell fate determination.** SP, Secretory progenitor; AP, absorptive progenitor. Notch and Wnt signaling are known to effect cell fate decision. Wnt activation induces early secretory programming. Although not linked to Wnt signaling, Math-1, a transcription factor, drives cells toward an initial secretory fate. Further expression of neurogenin-3 (ngn-3), Klf-4, and β-catenin induce terminal differentiation into enteroendocrine cell, goblet cells, and Paneth cells, respectively. Notch activated progenitor cells progress toward an absorptive fate through Hes-1 target gene expression. In fact, Hes-1 inhibits Math-1 expression, further strengthening this initial absorptive decision. (See color insert.)

phenotypes. The overexpression of Dkk1, a Wnt inhibitor, causes a loss of all secretory cell types, although absorptive enterocytes differentiate normally (Kuhnert et al., 2004; Pinto et al., 2003). In addition, nuclear β-catenin is required for the expression of functional Paneth cell molecules, including EphB3 and cryptdin/defensin (Andreu et al., 2005; Batlle et al., 2002).

Finally, Wnt signaling has been shown to be important in cellular migration and localization along the crypt–villus epithelium. Eph receptor molecules are targets of β-catenin–mediated gene expression, and Eph/Ephrin signals aid in compartmentalizing proliferative cells in the crypt from the nonproliferative, more differentiated cells along the villus (Batlle et al., 2002; Shih et al., 2001; van de Wetering et al., 2002). Cells expressing transmembrane ephrin ligands and adjacent cells expressing Eph receptors exhibit repulsive properties, and they have been shown to be important for maintaining cellular boundaries and establishing migratory paths (Xu et al., 1999). In fact, EphB2 and EphB3 have been shown to be expressed on progenitor and Paneth cells, respectively, whereas ephrin B1 (one of the Eph ligands) is expressed at the crypt–villus junction. The deletion of EphB2 and EphB3 results in the dispersion of Paneth cells, which are normally restricted to the bottom of the crypt, along the crypt–villus axis. Additionally, cells expressing ephrinB1, which are normally restricted to the crypt–villus junction, can be found within the crypt (Batlle et al., 2002). Thus, Wnt signaling through nuclear β-catenin drives the expression of EphB2 in crypt cells as well as of EphB3 in Paneth cells, thereby ensuring the proper segregation of the proliferative cells and the Paneth cells of the crypt from the differentiated cells of the villus (Batlle et al., 2002; van de Wetering et al., 2002).

B. Bone Morphogenic Protein Signaling

Although Wnt signaling stimulates stem cell and progenitor proliferation in the crypt, BMPs inhibit cellular division in the ISCs and the villus epithelium. BMP signaling occurs via the dimerization of type I and II BMP receptor proteins, which have intrinsic Ser/Thr kinase activity. After dimerization, type II phosphorylates type I, which results in the activation of receptor-regulated Smad (R-Smad) via phosphorylation. R-Smad then forms a heterodimer with Smad4, which is a common Smad. This R-Smad/Smad4 complex is translocated to the nucleus, and it can act as a transcriptional regulator, eliciting both the activation and repression of target gene expression through coactivator or corepressor binding (see Figure 44.2; Shi and Massague, 2003; see Chapter 1). The expression of BMP2 and BMP4 is limited to the mesenchymal tissue surrounding both the villus epithelium and the stem cells within the crypt (Hardwick et al., 2004). Additionally, BMPR1A has also been identified in ISCs as well as villus epithelial cells, but not in transient amplifying progenitors (He et al., 2004). Active BMP signaling has been confirmed in these BMPR1A^{+} cells by the identification of phosphorylated Smad1, Smad5, and Smad8 (these are R-Smads; He et al., 2004). Human genetic studies have reported BMPR1A and Smad4 mutations in juvenile polyposis, which is a syndrome that involves numerous hamartomatous polyps in the small intestine (Howe et al., 1998a; 1998b; 2001). Additionally, BMPR1A knockout mice and transgenic mice overexpressing the BMP antagonist Noggin both demonstrate an overabundance of crypt structures and enhanced Wnt/β-catenin activity (Haramis et al., 2004; He et al., 2004).

As indicated previously, two active BMP signaling regions have been identified: one in the villus epithelium and the other within the putative intestinal

stem cells in the crypt (He et al., 2004). BMP signaling within the villus region must remain within these terminally differentiated cells to inhibit ectopic crypt formation (Haramis et al., 2004). Although the exact mechanism remains unknown, BMP signaling can inhibit proliferation in ISCs perhaps in part through the Smad transcription factors and in part via interaction with PTEN. However, the inhibition of stem cell self-renewal via BMP signaling must be balanced by other permissive signals and coordinately regulated, thus implying a more intricate system.

C. PTEN/PI3K/Akt Signaling

Wnt signaling, which is measured by phosphorylated low density lipoprotein-receptor-related protein 6 (LRP6), is found in all proliferating crypt cells, including stem cells, whereas only a small number of these cells actually show β-catenin–driven transcriptional activity using a Top-Gal transgenic mouse model in which a LacZ reporter gene is driven by a TCF optimal promoter (He et al., 2004). Therefore, other signals may assist Wnt in the regulation of β-catenin, particularly in stem cells. Indeed, PTEN, which is already reported to control stem cell maintenance in the hematopoietic stem cell, may play a similar role in the intestine (Zhang et al., 2006). PTEN is a tumor suppressor that displays tyrosine phosphatase and lipid phosphatase activity. Functionally, PTEN antagonizes PI3 kinase (PI3K) activity, thereby inhibiting Akt, a Ser/Thr kinase that is the main downstream mediator of the PI3K pathway. PTEN cannot be recruited to the membrane to suppress PI3K after its phosphorylation (see Figure 44.2; Vazquez et al., 2001). Interestingly, mutations in PTEN have been identified in patients with Cowden disease and Bannayan–Zonana syndrome, both of which share intestinal polyp development (Liaw et al., 1997; Marsh et al., 1997). Because defects in BMP, PTEN, and Wnt signaling pathways can all result in similar intestinal pathology, it is possible that these signaling pathways comprise a coordinated regulatory mechanism for intestinal stem/progenitor cell regulation. Indeed, Akt has been shown to effect β-catenin transcriptional activity. Evidence indicates that Akt can phosphorylate β-catenin, directly promoting 14–3-3ζ binding and facilitating β-catenin stabilization (Tian et al., 2004). Indeed, phosphorylated PTEN has been identified along with activated Akt and 14–3-3ζ in ISCs (He et al., 2004). Additionally, active PTEN has been shown to decrease nuclear levels of β-catenin (Persad et al., 2001), whereas recent work has shown that loss of PTEN in the crypt epithelium and surrounding stroma coordinately results in an increased number of ISCs and progenitors displaying active Akt signaling, nuclear β-catenin, and cyclinD1 and correspondingly increased cell cycle activity. This evidence establishes the role of PTEN as a negative regulator of PI3K/Akt signaling in ISCs and progenitors. Because BMP signaling has been shown to positively regulate PTEN (Waite and Eng, 2003), whereas Noggin antagonizes BMP signaling, these two signals can be used to regulate PTEN control on Akt activity which assists Wnt signaling in controlling β-catenin activity within the ISC (He et al., 2004; He et al., 2007).

D. Notch Signaling

Signaling mechanisms regulating cell fate determination within the intestine are just as important as those controlling proliferation. They ensure an appropriate mixture of fully differentiated cell types to facilitate proper intestinal

epithelial function. Notch signaling contributes to the decision-making apparatus developed to enable appropriate cellular specialization.

Notch signaling occurs through the interaction of transmembrane molecules on adjacent cells. Delta and Jagged family transmembrane ligands on one cell can activate Notch receptors on an adjacent cell (Artavanis-Tsakonas et al., 1999). This activation results in a series of unique events involving the transmembrane and intracellular domains of the single-pass transmembrane Notch receptor. Upon ligand binding, γ-secretase activity within the membrane cleaves the intracellular domain, thus freeing it from the cytoplasmic surface. The intracellular domain of Notch (NICD) translocates to the nucleus, where it binds CSL/RBPJκ and activates target gene expression, including the expression of other transcriptional regulators such as Hes family proteins (see Figure 44.2; Artavanis-Tsakonas et al., 1999).

Virtually all Notch expression in the intestine is limited to the crypt epithelium, which suggests that Notch signaling occurs among crypt cells (Schroder and Gossler, 2002). Logically, cell fate decisions would need to be made within the crypt progenitor population so that after cells migrated to the villus, they would already be programmed with a specific cell fate. Because two lineages exist within the intestinal epithelium (one secretory and the other absorptive), it stands to reason that the first fate decision is between these two pathways (see Figure 44.3). Evidence for Notch signaling in this initial determination has come from studies in which Notch pathway intermediates have been mutated or deleted. Notch inhibition via the deletion of CSL/RBPJκ or treatment with γ-secretase inhibitor results in an intestinal epithelium composed almost entirely of goblet cells (Milano et al., 2004; van Es et al., 2005). The inhibition of Notch signaling by deleting Hes also causes an overabundance of goblet and enteroendocrine cells (Jensen et al., 2000). Conversely, the constitutive expression of NICD results in an epithelium that is devoid of all secretory cells, including Paneth cells (Fre et al., 2005). Thus, Notch-activated cells are inhibited from progressing down the secretory lineage, and they are destined to become enterocytes. Furthermore, secretory cells avoid Notch signaling by expressing Notch ligands and inducing Notch activation in adjacent cells, thereby inhibiting neighboring cells from differentiating down a similar pathway and ensuring an appropriate ratio of secretory and absorptive cells (Crosnier et al., 2005).

Mouse atonal homologue 1 (Math1) is a transcription factor that is expressed in secretory progenitors. Math1 mutant mice display a phenotype that is similar to that of transgenic NICD mice in that they have a complete lack of secretory cell types, although absorptive enterocytes develop normally (Yang et al., 2001). Hes1 in intestinal progenitor cells inhibits Math1 expression by inducing an absorptive fate determination (Jensen et al., 2000). Thus, $Notch^{+}/Math1^{-}$ cells become absorptive enterocytes, whereas $Notch^{-}/Math1^{+}$ cells progress toward a secretory fate. Furthermore, other factors are involved in later secretory fate decisions. Neurogenin-3 mutant mice lack enteroendocrine cells, but they contain all other cell types, which indicates that this transcription factor pushes development toward enteroendocrine cells that can specialize even further on the basis of the molecules that they are induced to produce and secrete (Jenny et al., 2002). Goblet cells express Kruppel-like factor 4, which, on the basis of Kruppel-like factor 4 mutant experiments, is important in the differentiation of this secretory cell type (Katz et al., 2002). Thus, although a complete understanding of the signals providing cues for

cellular fate decisions is lacking, our knowledge about these decision-making processes has grown tremendously during recent years (see Figure 44.3).

Finally, Wnt and Notch pathways may also be linked, and this provides more evidence for multiple regulatory signaling mechanisms controlling stem cell maintenance as well as intestinal differentiation. Interestingly, when APC^{min} mice bearing a germline mutation in APC are treated with a γ-secretase inhibitor to block Notch signaling, not only do all the intestinal epithelial cells become secretory in nature, but proliferation within the crypt ceases (van Es et al., 2005). In addition, if Notch signaling is overactive in an area of inactive Wnt signaling, proliferation is absent (Zecchini et al., 2005). Finally, increases in proliferative cell number occur when Notch is constitutively activated in areas of canonical Wnt activity within the crypt (Fre et al., 2005). Thus, it would seem that both Notch and Wnt signaling together are required for driving the proliferation of ISC/progenitor cells and for the control of appropriate cell fate determination.

V. INTESTINAL STEM CELLS IN HUMAN DISEASE

A. Stem Cells in Colorectal Cancer

Genetic analyses of sporadic and colitis-induced colorectal cancer (CRC) tissues indicate that genes regulating essential functions of intestinal stem cells are frequently mutated early during neoplastic transformation (Radtke and Clevers, 2005). The model of colonic neoplastic transformation proposed by Fearon and Vogelstein (1990; Figure 44.4) suggests that CRC results from gene mutations and chromosomal instability that occur in a preferred sequence (Radtke and Clevers, 2005). Mutations occur in genes that regulate the self-renewal, proliferation, and cell fate decisions of ISCs. Early mutations

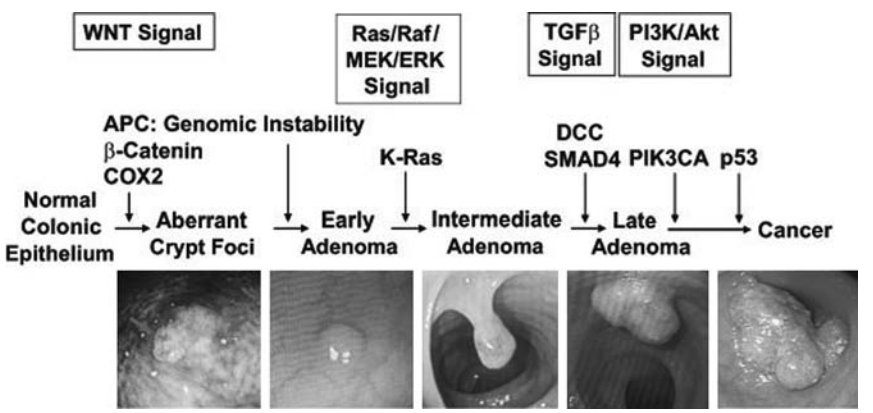

FIGURE 44.4 **Intestinal carcinoma development.** This figure outlines the proposed model for tumorigenesis within the intestine, along with corresponding human colonoscopic images. Progression proceeds as various genetic mutations accumulate in a preferred sequence. Initially, mutations in Wnt pathway components such as APC, β-catenin, and Cox-2 lead to aberrant crypt foci. These ectopic crypts, outlined with an indigo carmine dye, can be detected via chromoendoscopy. APC mutations can also result in genomic instability, leading to development of an early adenoma. Further mutations in MAP kinase signaling proteins like K-Ras and then TGFβ pathway components, such as DCC (deleted in colorectal carcinoma) and Smad4 genes result in progression toward an intermediate and late adenoma, respectively. Finally, mutations in PI3K subunits (i.e., PIK3CA) and p53 lead to cancer, shown here as a locally invasive exophytic carcinoma. (See color insert.)

of APC enhance the risk for other mutations by increasing genomic instability. As lesions advance, their severity correlates with additive mutations in K-ras, Smad4, and p53. The combination of these effects endows tumor cells with enhanced proliferation, resistance to apoptosis, and the capacity for local invasion and metastatic spread. At this time, it is unclear whether these neoplastic alterations (e.g., constitutive Wnt/β-catenin signaling) act through rare stem cells or promote the dedifferentiation of progenitor cells. In addition, clarification is needed regarding the behavior of activated intestinal stem cells and cancer stem cells that emerge in CRC.

The earliest mutation associated with CRC development involves the APC gene. APC is mutated in familial adenomatous polyposis and in 85% of sporadic CRC (Grady and Markowitz, 2002; Miyaki et al., 1994). The mutational inactivation of APC causes epithelial hyperproliferation and precancerous aberrant crypt foci (Jen et al., 1994), which is the earliest lesion in colonic neoplasia. Thus, the subsequent induction of β-catenin target genes such as goblet-cell–associated intestinal trefoil factor and mucin 2 or Paneth cell matrilysin (matrix metalloproteinase 7) and cryptdin-1 may explain why some CRCs express "tumor markers" associated with secretory lineage cells (Blank et al., 1994).

Alterations of Wnt/β-catenin signaling by mutated proteins other than APC provide clues to the early pathogenesis of CRC. For example, mutations in β-catenin that prevent degradation by GSK3β are less likely to progress to large adenomas and CRC than lesions with APC mutations (Samowitz et al., 1999). One explanation for the difference in outcome may relate to data that suggest that loss of APC increases genomic instability. Thus, lesions harboring APC mutations also carry the risk for polyploidization and chromosomal instability (Fodde et al., 2001; Kaplan et al., 2001). The impact of this effect is compounded by the localization of APC mutations and subsequent chromosomal alterations in long-lived cancer stem cells. Similar additive effects of mutations in K-ras and p53 have been described. Both genes are mutated in 50% of human CRCs. Recent results from murine models indicate that the transgenic expression of an oncogenic K-ras mutation *(K-rasV12G)* causes aberrant crypt foci with subsequent invasive adenocarcinoma (Janssen et al., 2002). Interestingly, more than 40% of K-rasV12G tumors harbored inactivating mutations in p53 or demonstrated a loss of heterozygosity (LOH; i.e., a loss of the wild-type allele). These data and others (Lin and Lowe, 2001) indicate that cross-talk between oncogenic Ras and p53 pathways contributes to CRC progression. However, lesions that harbor APC mutations before accumulating Ras and p53 mutations appear to carry a worse prognosis, and this is possibly related to the chromosomal instability associated with APC mutations.

The existence of cancer stem cells in CRC has recently gained support. However, the origin of mutated stem cells in intestinal neoplasia continues to spark considerable debate among researchers. The "top-down" model formulated by Fearon and Vogelstein proposes that mutant cells originate in the upper crypt and surface epithelia of adenomas (Fearon and Volgelstein, 1990). In these studies, researchers reviewed tissue morphology, Ki-67 staining (a proliferative marker), nuclear β-catenin staining, and single nucleotide polymorphism analyses of DNA from microdissected cells to detect APC mutations and LOH in small (<3 mm) colorectal adenomas. They found an overwhelming preponderance of epithelial dysplasia harboring APC mutations localized to intercryptal surface epithelium, and they proposed that

dysplastic cells spread across the colonic surface and downward to displace adjacent crypts. These data support the notion that mutant stem cells initiate dysplasia from a position between crypt orifices. However, the data do not exclude the possibility that rare transformed stem cells originate in crypt bases and migrate to intercryptal areas, where they expand onto other surfaces and into other crypts from above.

The "bottom-up" paradigm of colorectal adenomas states that lesions begin in stem cells within a more classic stem cell niche near the crypt base. Cells that accumulate genetic alterations (e.g., mutations, LOH) in key regulatory pathways acquire a selective advantage and expand to colonize the entire crypt; this is referred to as a *monocryptal adenoma*. Evidence for this pathway was presented in work by Novelli et al. (1996), who used *in situ* hybridization for the Y chromosome in a naturally occurring XO/XY patient with FAP. By defining the karyotype of individual cells within adenomas, they detected evidence for the spread of mutated stem cell clones by crypt fission (splitting at the base), with the subsequent "bottom-up" conversion of adjacent crypts. Thus, the progeny of mutated clones expand, either stochastically or as a result of a selective advantage (e.g,. greater proliferative capacity) to spread the same disordered genetic code to other essential crypt stem cells. More recent findings in genetically altered animal models suggest that crypt fission as well as crypt budding may be regulated by the PI3K pathway. In this work, loss of PTEN led to abnormal activation of PI3K/Akt pathways that, in cooperation with Wnt signaling, resulted in cancer stem cells and subsequent polyp formation. Closer inspection of the polyps revealed that these cancer stem cells were initiating both crypt fission and budding, two processes normally found only in fetal and neonatal stages, supporting cancer stem cell involvement in both the "top-down" and "bottom-up" crypt formation (He et al., 2007). At present, data continue to suggest that, in larger adenomas (i.e., those carrying multiple mutations), the progeny of mutated cells descend into crypts from the surface in a "top-down" fashion. However, in early lesions, mutated stem cells originate in or near niches that are close to the crypt base. Mutated progeny generate a monocryptal adenoma in a "bottom-up" fashion, but these spread to adjacent areas by crypt fission. The "top-down" model, then, likely applies to more advanced lesions (i.e., larger adenomas and cancer), whereas the "bottom-up" processes best explain events in aberrant crypt foci and monocryptal adenomas (Leedham et al., 2005).

B. Stem Cell Changes in Colitis-Induced Cancer

Data generated in cancers arising in patients with ulcerative colitis (UC) and Crohn's disease (CD) suggest that inflammation alters the sequence of transforming events in CRC. Additionally, recent work has linked inflammation with increased crypt fission leading to inflammatory based colon cancer (Chen et al., 2005). UC and CD, which are collectively referred to as *inflammatory bowel diseases* (IBDs), increase the risk for CRC by 10-fold as compared with the rate seen among healthy cohorts (Itzkowitz and Yio, 2004). The greatest risk is associated with patients with extensive inflammation and ulceration (Ekbom et al., 1990). As the colon repairs areas of ulceration, new crypts are generated in the setting of oxidative and nitrosative stress, and this leads to DNA damage and an increased cancer risk (Hussain et al., 2003; Seril et al., 2003). DNA alterations are initiated in colonic crypts and expand to

adjacent crypts through crypt fission (Chen et al., 2005). A major early mutational target linked to the early onset of dysplasia and progression to cancer appears to be the p53 gene (Staib et al., 2005). Mutations of p53 enhance clonal expansion and chromosomal instability through effects on cell cycle checkpoints and apoptosis (Robles and Harris, 2001). Examination of UC tissue indicates that *p53* mutations are a relatively early event in IBD-associated CRC as compared with sporadic CRC, in which p53 mutations occur late (Brentnall et al., 1994; Itzkowitz and Yio, 2004). Genomic instability may be an important outcome of inflammation-induced p53 mutation and DNA damage, because genomic instability has been detected in up to 36% of nondysplastic areas and 86% of dysplastic areas in patients with UC (Willenbucher et al., 1999). Brentnall et al. examined genomic instability among patients with UC with dysplasia (progressors; Brentnall, 2003) and those with UC who did not have dysplasia and who were free of cancer (nonprogressors; Chen et al., 2003). They confirmed the widespread incidence of genomic instability in patients with UC. More importantly, they used DNA fingerprinting to indicate that genomic instability was present in more areas taken from progressors than nonprogressors. An important feature of these studies was the detection of genomic instability in nondysplastic tissue found in patients with known dysplasia; this suggests that genomic instability precedes neoplastic transformation in IBD.

The challenge of understanding IBD-associated CRC has led to several recent novel observations with far-reaching implications. Whereas IBD is associated with rapid cell turnover, Rabinovitch et al. hypothesized that telomere shortening may be accelerated (O'Sullivan et al., 2002). Because telomeres help prevent chromosome bridge breakage, telomere shortening has been associated with chromosomal losses (Gisselsson et al., 2001). A surprising result of these studies was the detection of telomere shortening in nondysplastic regions (e.g., rectum) taken from patients with known dysplasia. Because stem cell regeneration (occurring in areas of repair) requires that DNA errors be kept to a minimum, these data suggest that chronic inflammation induces an organ-wide risk for neoplastic transformation. These results also support the notion that the detection of genomic instability and telomere shortening may be useful biomarkers for patients involved in cancer surveillance programs.

VI. CLOSING REMARKS

In summary, ISCs reside near the crypt base along with adjacent specialized mesenchymal cells that comprise a specialized microenvironment (niche). The interaction between these two cell types is responsible for controlling initial stem cell division. As a result of this division, one stem cell remains attached to the niche (self-renewal), whereas the daughter stem cell, which is now within a different microenvironment, continues to receive activating signals that lead to the development of varied progenitor populations. Several of these interactions have been discovered, including Wnt, BMP, PI3K, and Notch signaling pathways. Although Wnt-induced β-catenin transcriptional activation provides one permissive signal for ISC proliferation, signals through BMP and PI3K/Akt pathways regulate this activation by either inhibiting or enhancing Wnt-induced β-catenin activity. Although it is not well defined, Notch signaling may also be required for ISC/progenitor cell proliferation. In addition, Wnt and Notch signaling are involved in the determination of cell fate.

Wnt signals initiate secretory lineage programming, including driving the terminal differentiation of Paneth cells. Notch signaling inhibits secretory lineage commitment and thus drives an absorptive decision. The integrity of these signaling interactions is critical to the appropriate functioning of ISCs.

Mutations resulting in the inappropriate functioning of these pathways can result in unabated stem cell activation, thus providing a mechanism for initiating intestinal carcinogenesis. The importance of Wnt, BMP, PI3K, and Notch signaling in tumor formation is demonstrated by the various mutant mouse models discussed previously. Furthermore, sporadic intestinal adenomas and pathologies related to genetic diseases in humans have been shown to display specific defects. For example, somatic mutations in APC are found in an overwhelming majority of human intestinal tumors, whereas germline APC mutations are a definitive genetic characteristic of FAP. Additionally, BMPR1A and Smad4 mutations are associated with juvenile polyposis, and PTEN mutations are linked with Cowden disease. Understanding the effects of these genetic changes on normal intestinal regulation could enable interventions that are specifically targeted at aberrant pathways. For example, Notch pathway inhibitors, which have been shown to inhibit unrestricted Wnt activated crypt proliferation in APC^{min} mice, could be used to treat human intestinal tumors.

Finally, both developing intestinal epithelium and adult intestinal regeneration share an ability to produce the entire intestinal epithelial cell repertoire, and this feature is driven by stem cells. Intestinal cancer, which is characterized by uncontrolled cell growth, is proposed to be governed by cancer stem cells. Thus, further research involving the roles of stem cells in development, regeneration, and tumorigenesis will provide a clarification of the mechanisms that regulate cellular proliferation and differentiation in the intestine, and this will ultimately lead to novel therapeutic strategies for fighting intestinal disease.

SUMMARY

- Intestine provides an ideal model for studying the regulation of adult stem cells.
- ISCs are responsible for intestinal regeneration.
- Niche interactions inhibit differentiation and control the self-renewal and proliferation of stem cells.
- Wnt, BMP, PI3K, and Notch signaling pathways are involved in the regulation of ISC proliferation and differentiation.
- Mutations in ISCs are possibly responsible for the origin of intestinal cancers.

GLOSSARY

Aberrant crypt foci
Uncontrolled crypt proliferation representing early lesions in the development of intestinal tumors.

Bromodeoxyuridine (BrdU)
A thymidine analog that can be incorporated into DNA; used to label cycling cells.

Colorectal cancer (CRC)
The most common type of gastrointestinal cancer.

Familial adenomatous polyposis (FAP)
An autosomal-dominant syndrome that predisposes an individual to the development of large numbers of colorectal polyps earlier in life.

Hamartomatous polyps
Benign polyps that consist of a disorganized collection of cells that are normally present in the intestine.

Inflammatory bowel disease (IBD)
A disease of chronic intestinal inflammation consisting of two main types: ulcerative colitis and Crohn's disease.

Invasive exophytic carcinoma
The pathologic description of a cancer that protrudes into the intestinal lumen (exophytic) and displays invasion into the submucosa.

Single nucleotide polymorphism analysis
A useful technique for finding single nucleotide changes in genes that may relate to disease.

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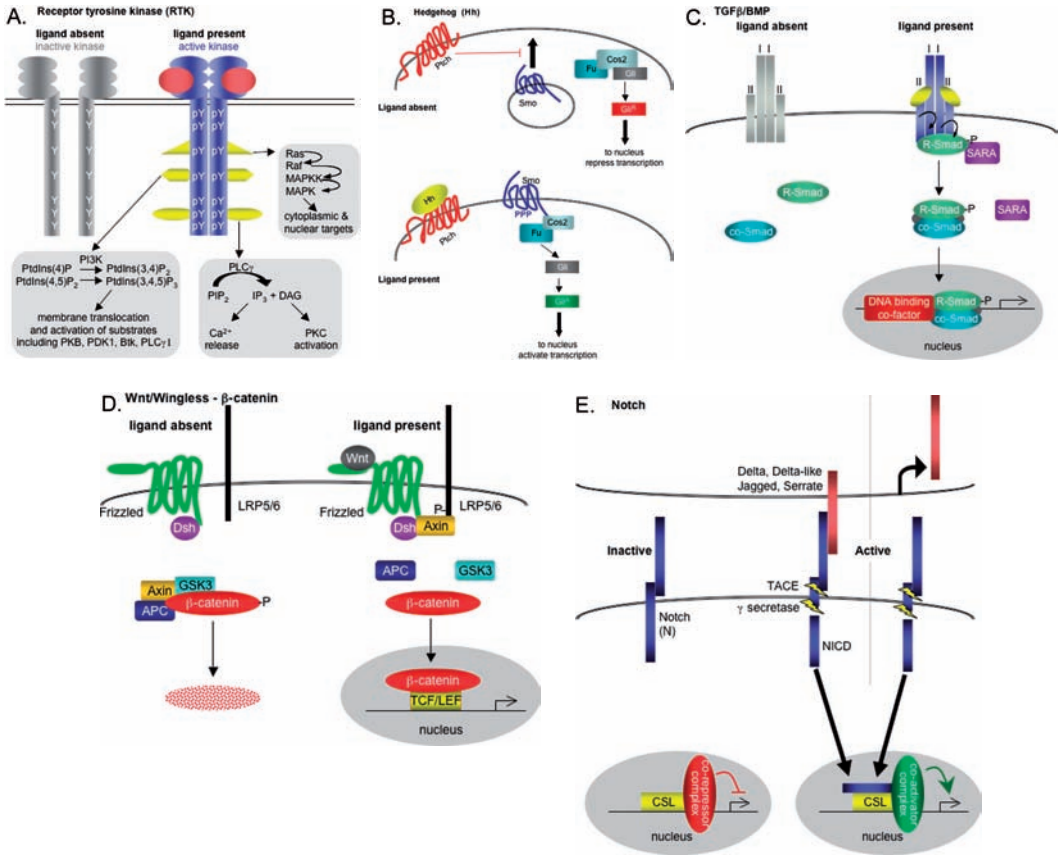


FIGURE 1.1

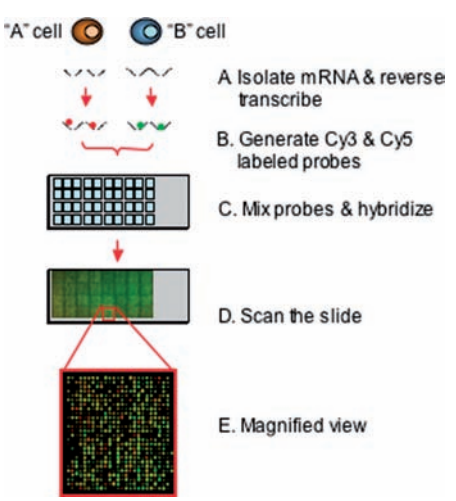

"A" cell
"B" cell
A Isolate mRNA & reverse transcribe
B. Generate Cy3 & Cy5 labeled probes
C. Mix probes & hybridize
D. Scan the slide
E. Magnified view

FIGURE 2.1

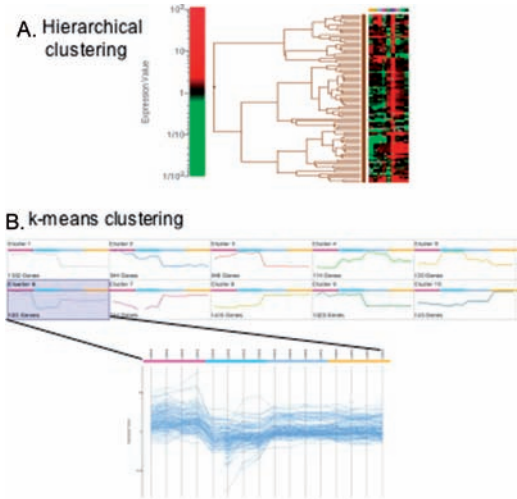


FIGURE 2.3

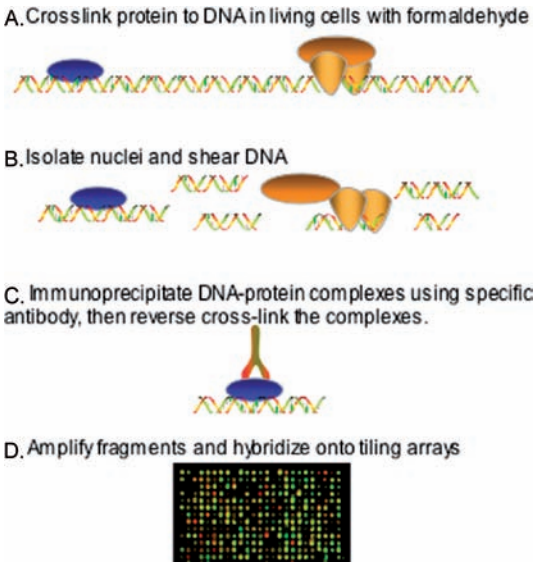

A. Crosslink protein to DNA in living cells with formaldehyde
B. Isolate nuclei and shear DNA
C. Immunoprecipitate DNA-protein complexes using specific antibody, then reverse cross-link the complexes.
D. Amplify fragments and hybridize onto tiling arrays

FIGURE 2.4

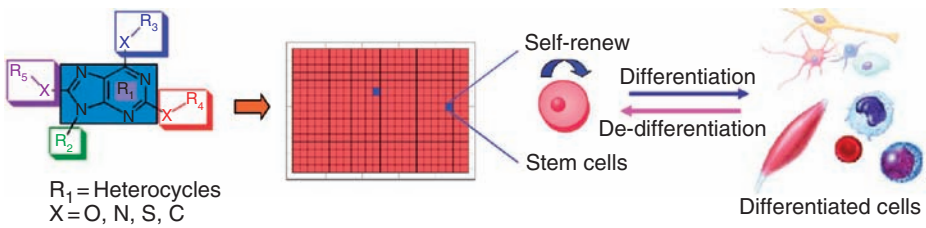
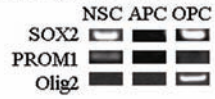


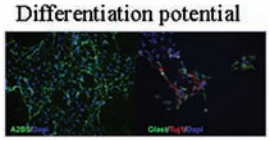
FIGURE 3.2

a. Sample verification

Marker gene expression



Differentiation potential



b. No. of genes detected in samples

Sample	No. of gene≥0.95
NSC	10087
APC	10283
OPC	11750

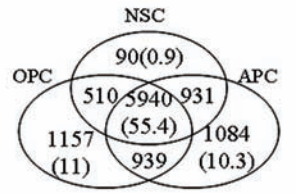
c. Accumulated gene No.(%) based on signal intensity

Signal Intensity	NSC	APC	OPC
<50	10551	10769	12386
≥50	11649	11431	9814
≥100	9438	9326	8016
≥500	3986	4300	4001
≥1000	2195	2642	2477
≥5000	313	508	396
≥10000	129	207	141
Total	22200	22200	22200

d. Correlation coefficient (R^2 score) among samples

	NSC	APC	OPC
NSC	1.00		
APC	0.65	1.00	
OPC	0.77	0.63	1.00

e. Distribution of genes among samples based on detection. No.(%)



f. Verify selected gene using independent methods

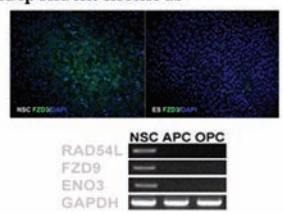


FIGURE 4.3

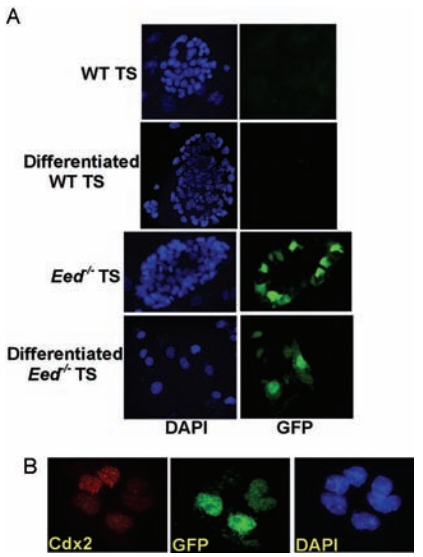


FIGURE 5.3

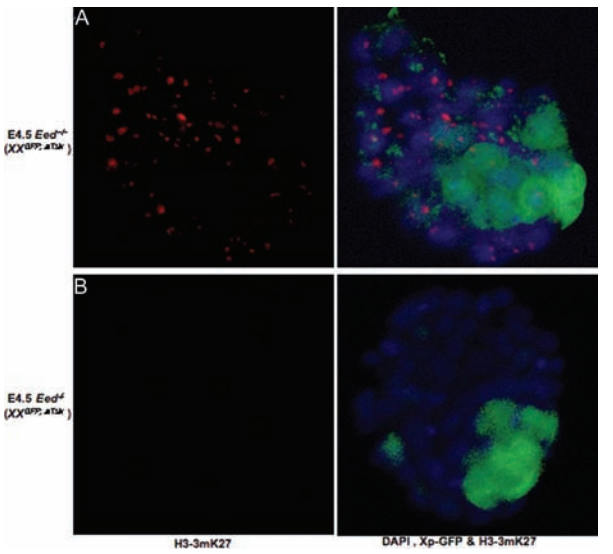


FIGURE 5.4

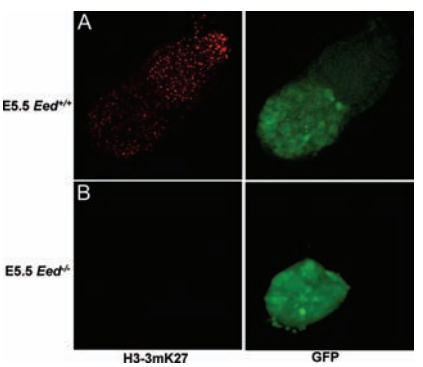


FIGURE 5.5

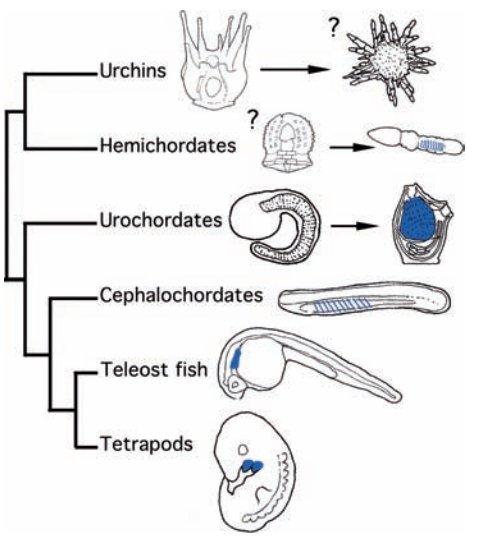


FIGURE 6.4

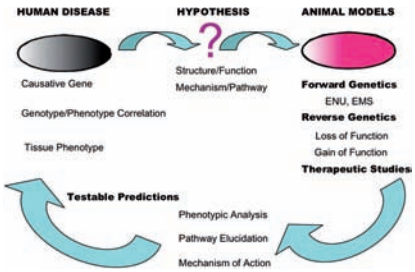


FIGURE 7.1

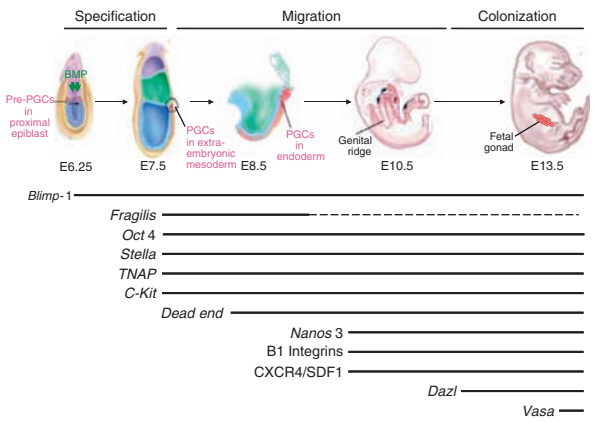


FIGURE 8.2

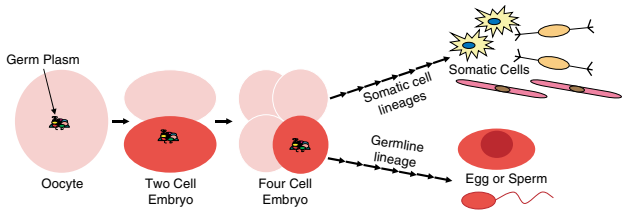


FIGURE 8.1

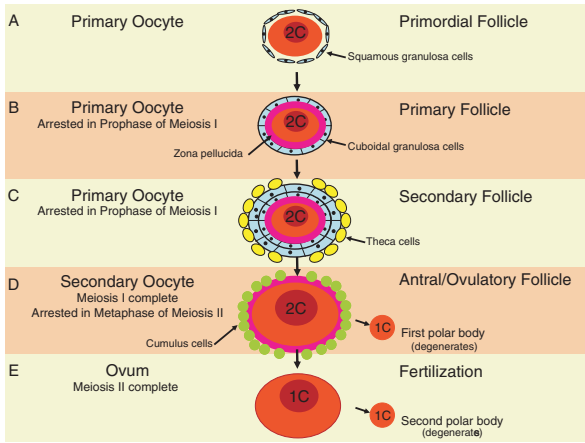


FIGURE 8.3

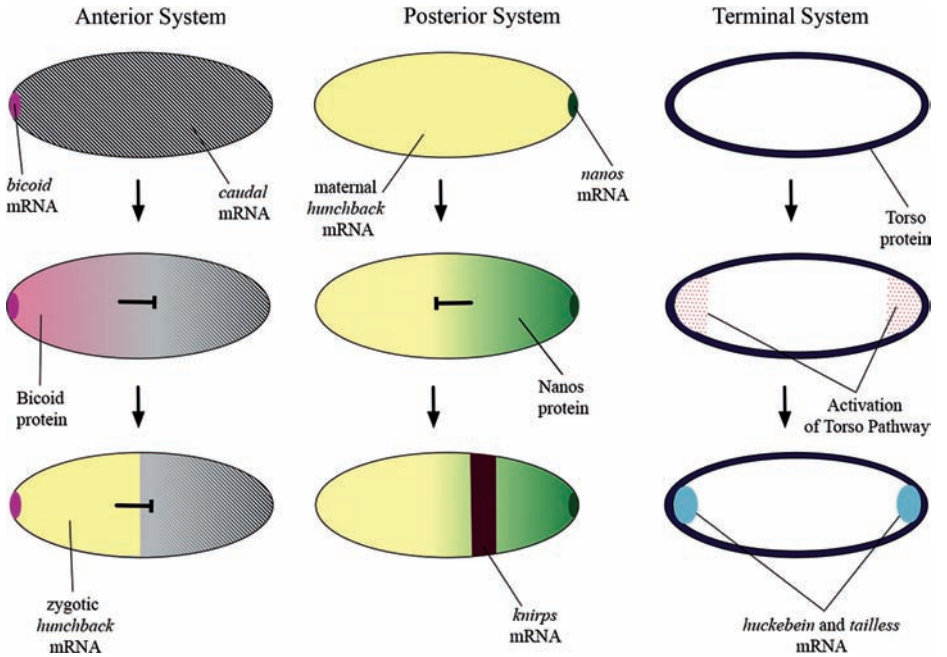


FIGURE 9.2

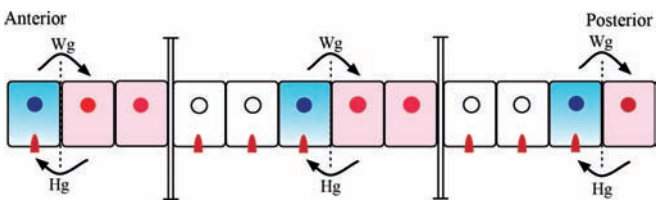


FIGURE 9.5

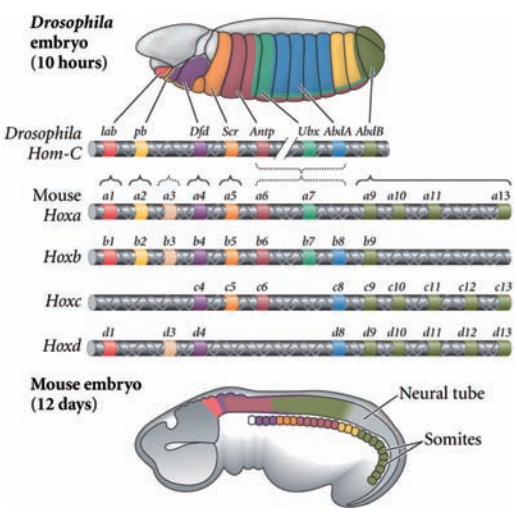


FIGURE 9.6

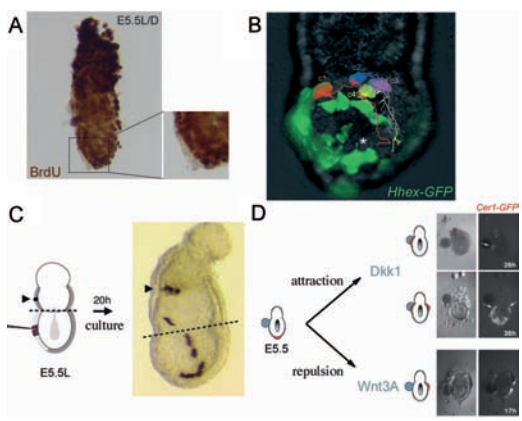
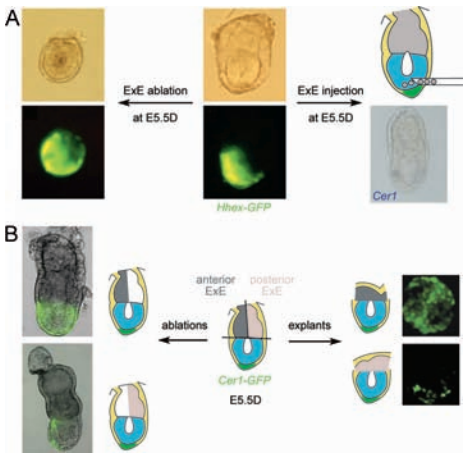


FIGURE 10.2



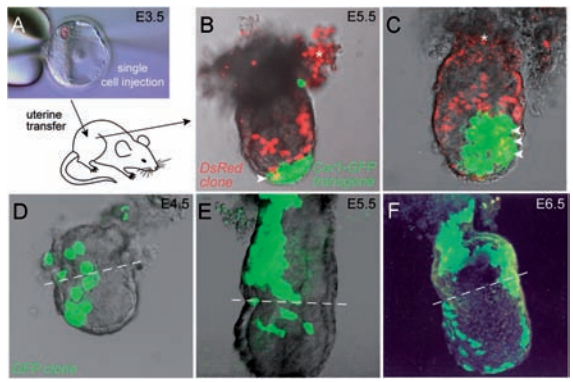


FIGURE 10.4

FIGURE 10.3

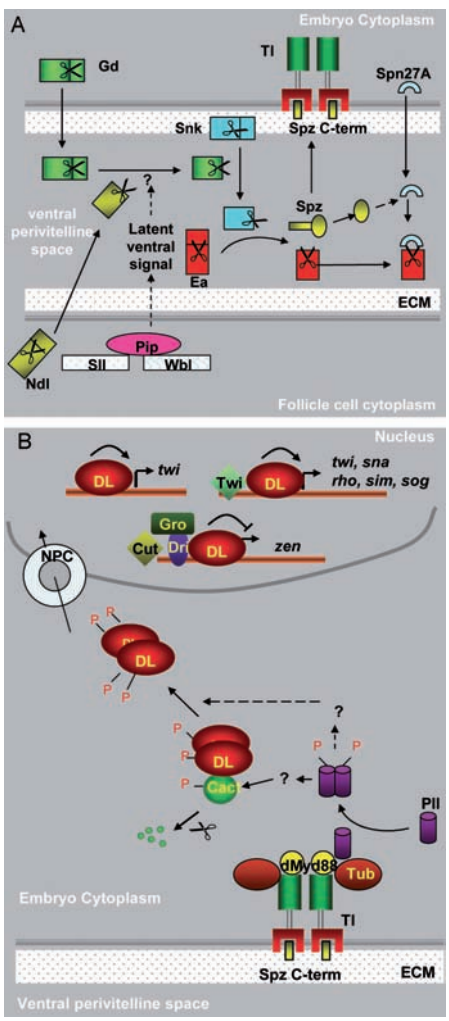


FIGURE 11.2

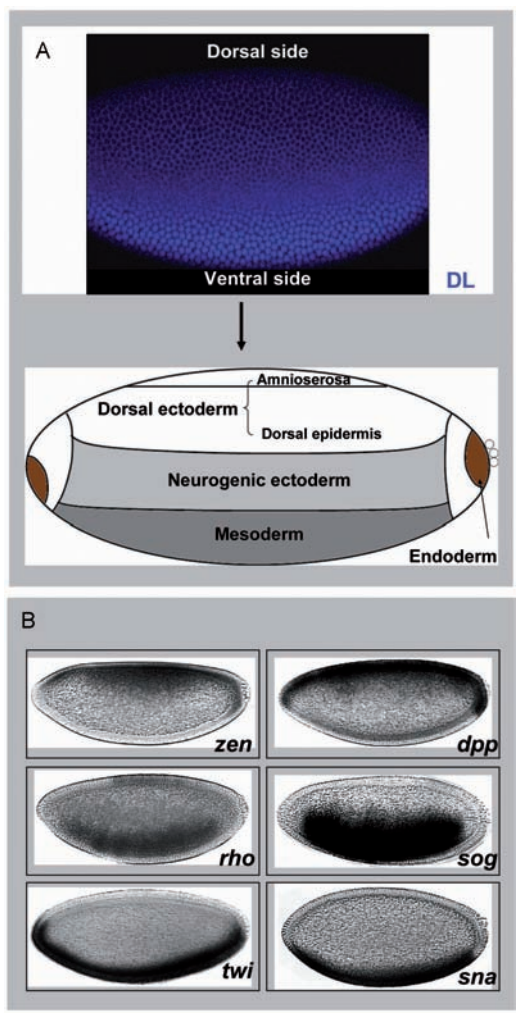


FIGURE 11.3

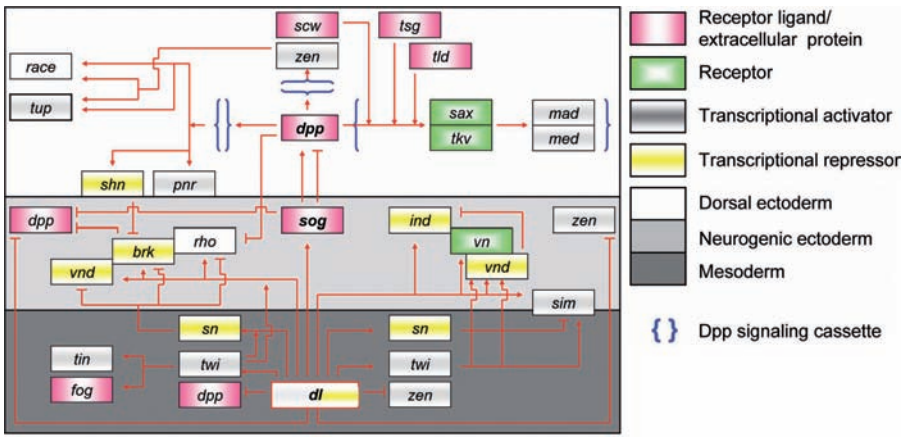


FIGURE 11.5

FIGURE 12.3

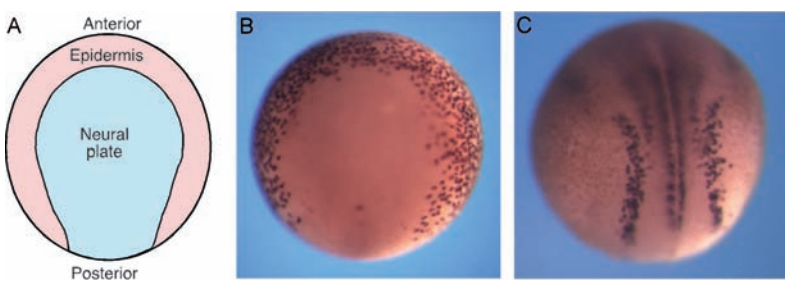


FIGURE 12.5

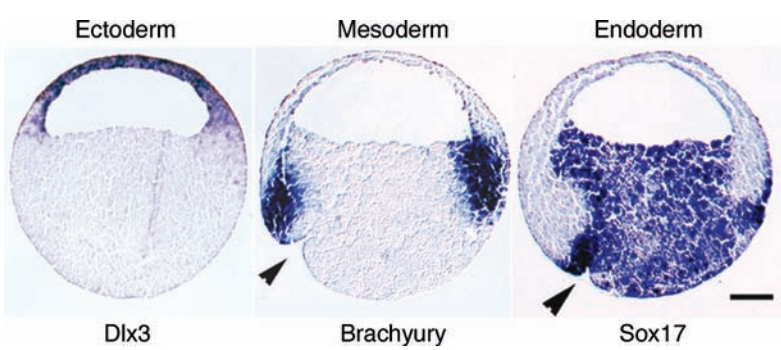


FIGURE 13.1

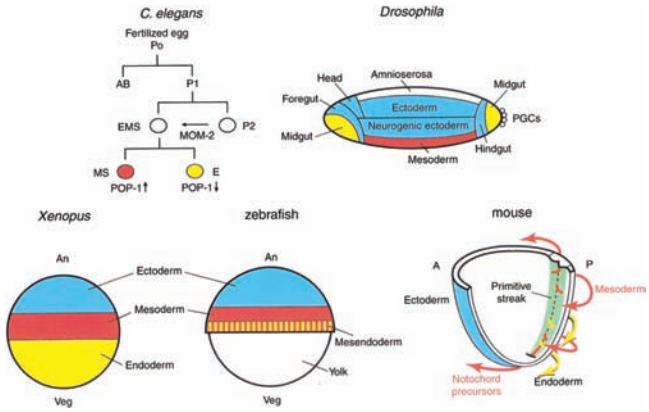


FIGURE 13.2

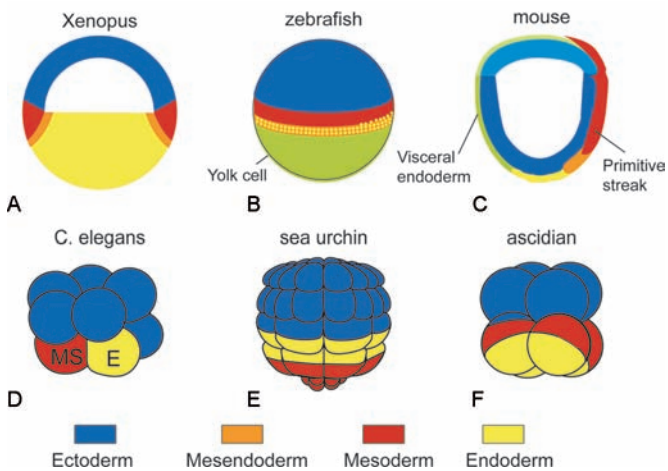


FIGURE 14.2

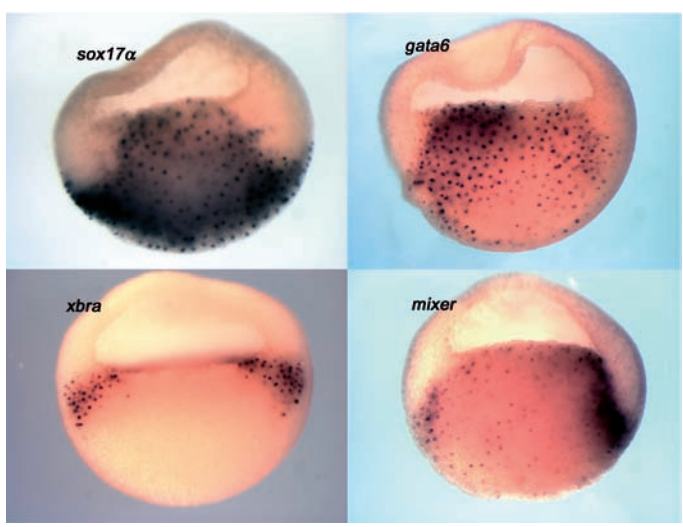


FIGURE 14.4

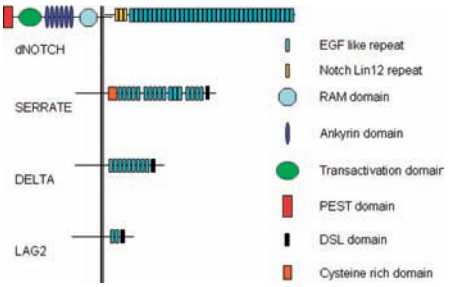


FIGURE 15.1

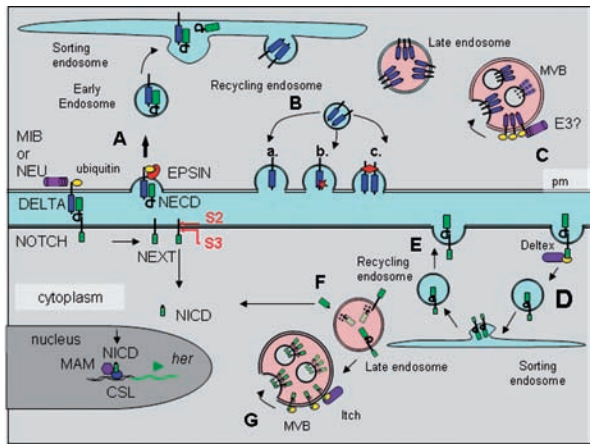


FIGURE 15.3

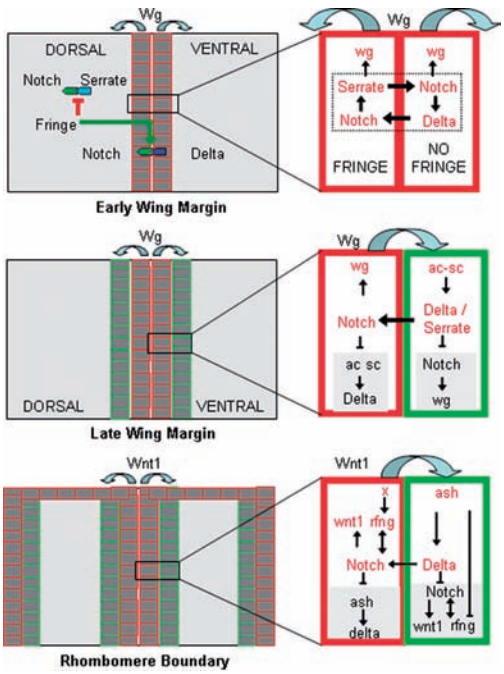


FIGURE 15.7

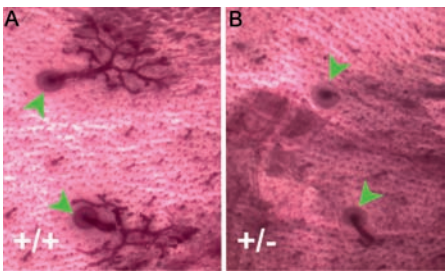


FIGURE 16.2

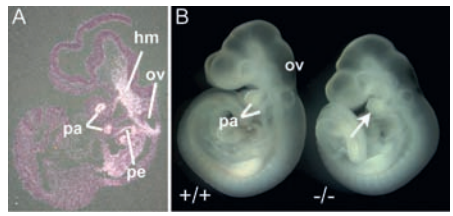


FIGURE 16.3

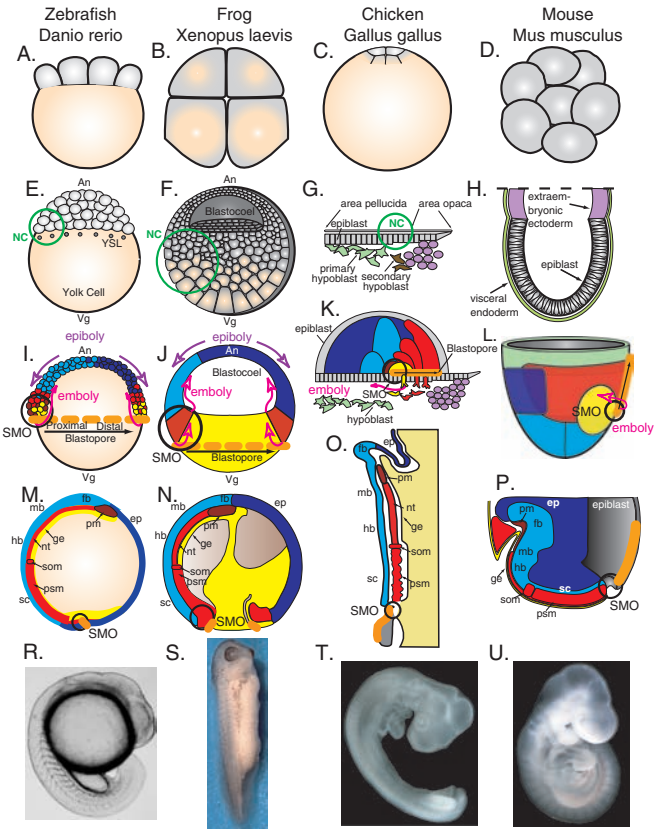


FIGURE 17.1

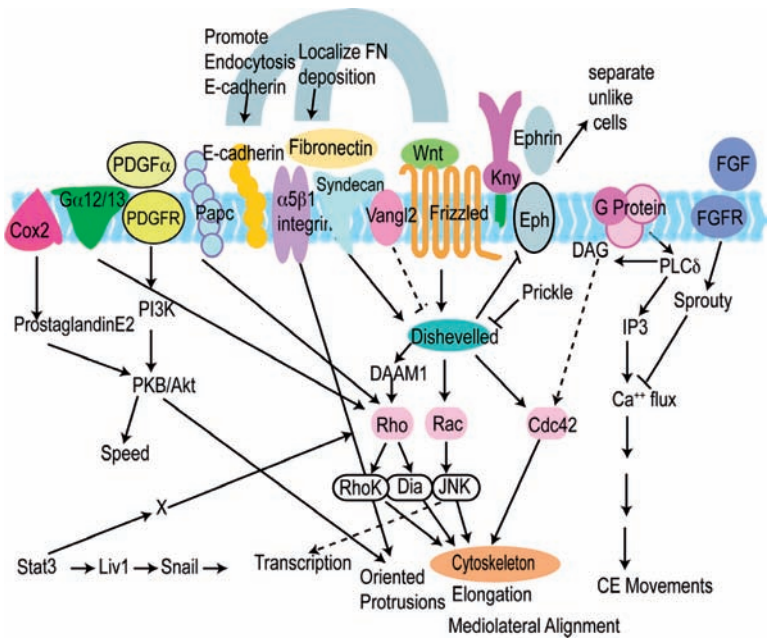


FIGURE 17.7

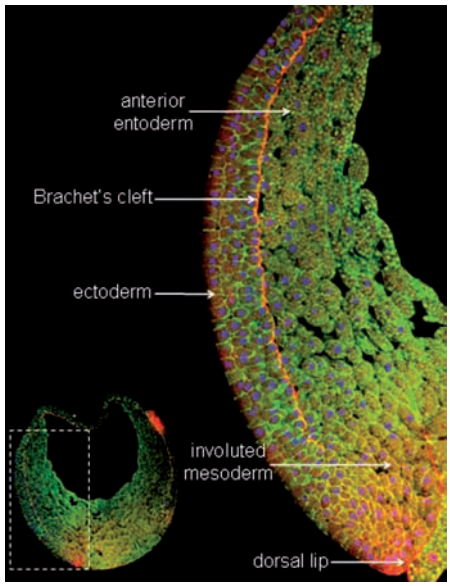


FIGURE 18.1

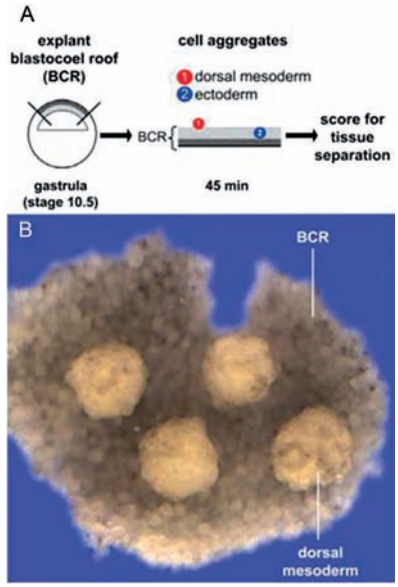


FIGURE 18.2

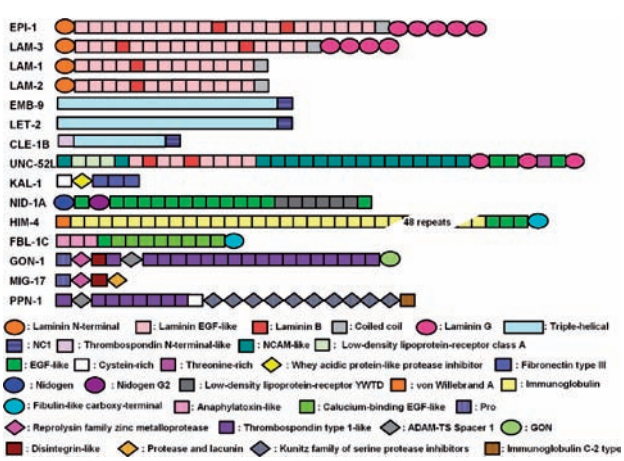
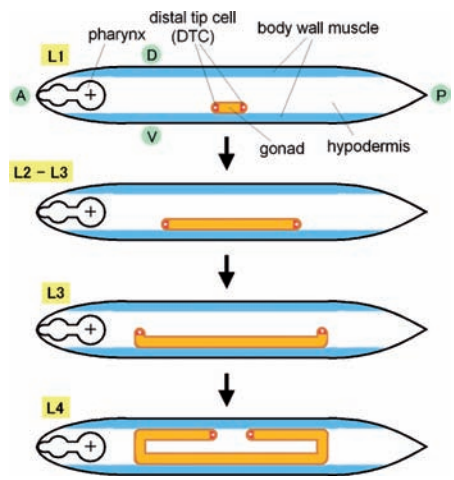


FIGURE 19.1



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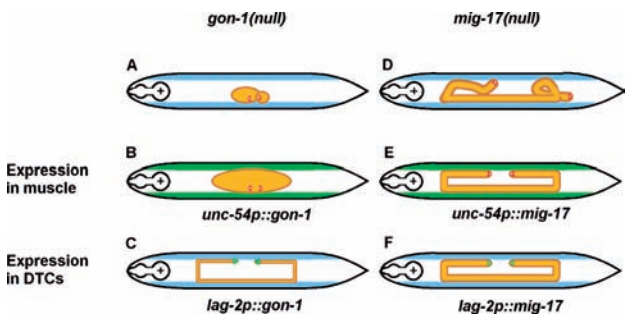


FIGURE 19.4

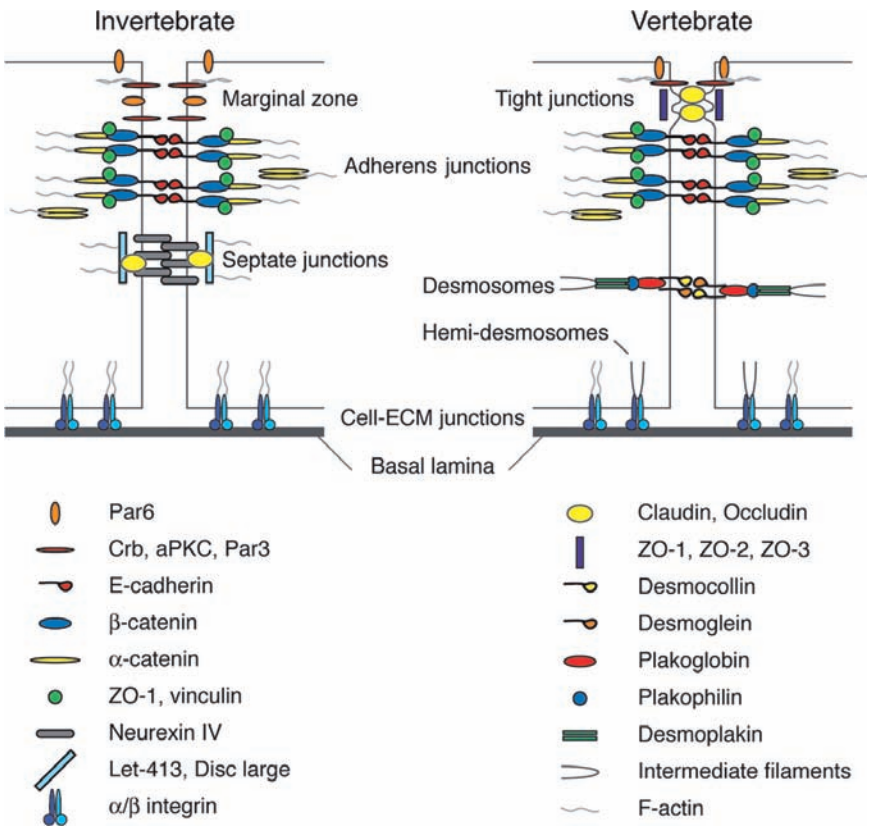
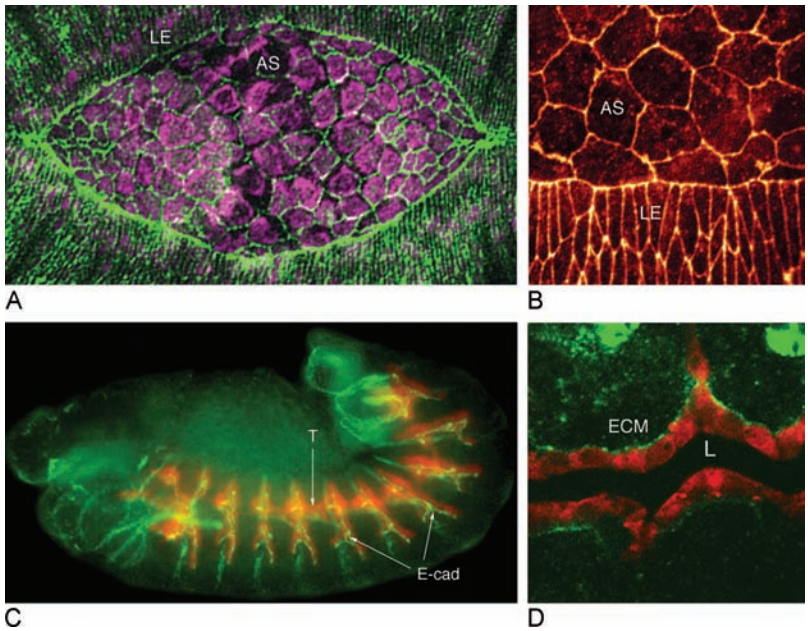


FIGURE 20.2



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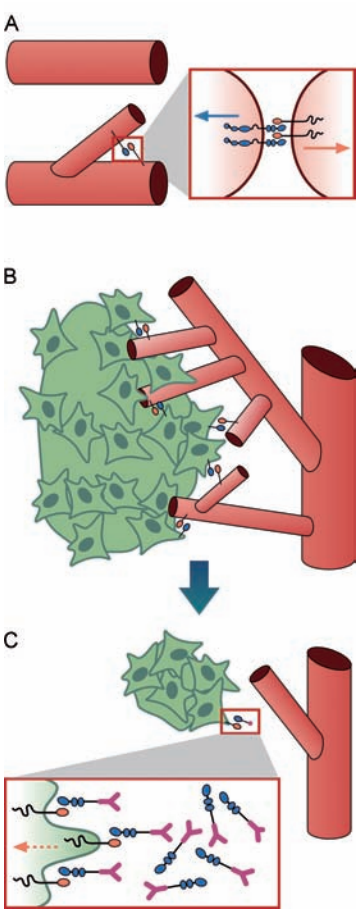


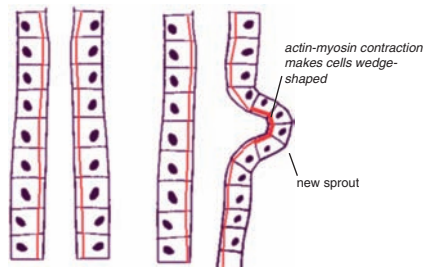


FIGURE 21.3

FIGURE 22.4

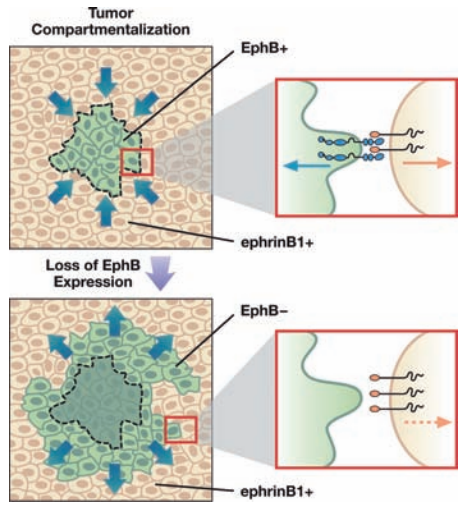


FIGURE 22.5

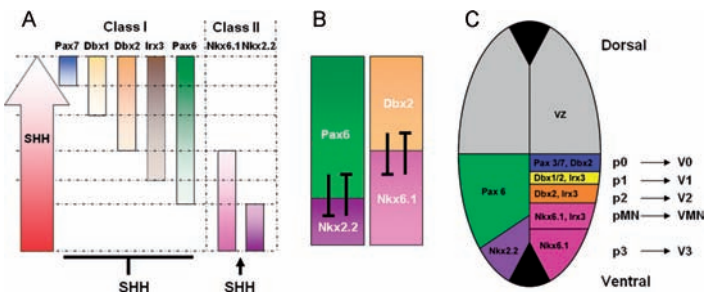


FIGURE 23.1

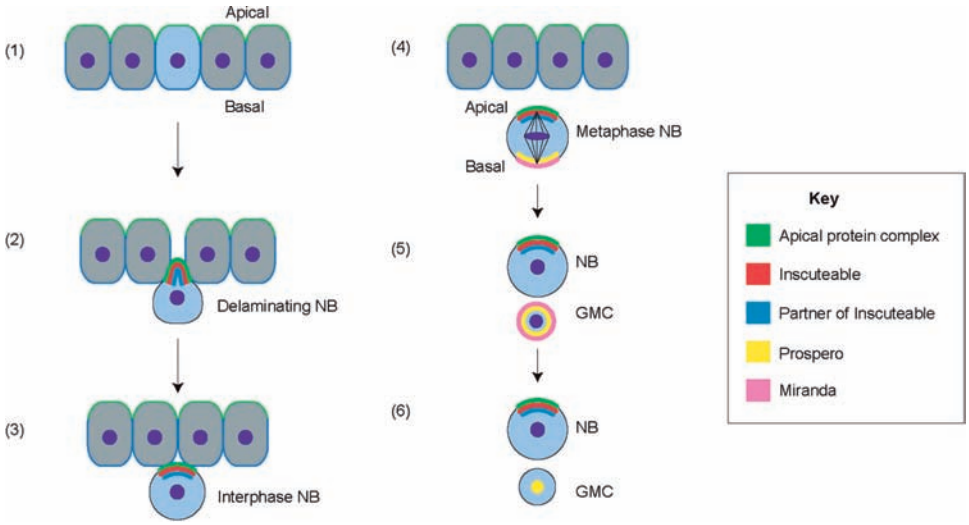


FIGURE 23.2

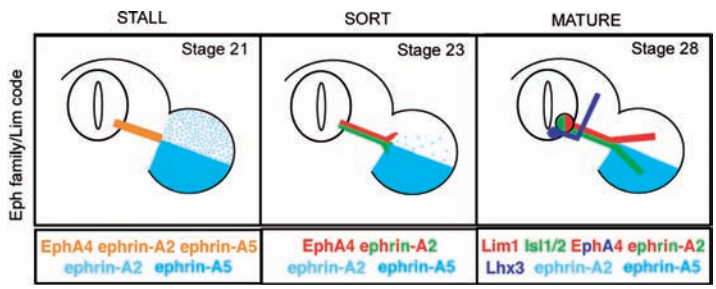


FIGURE 24.5

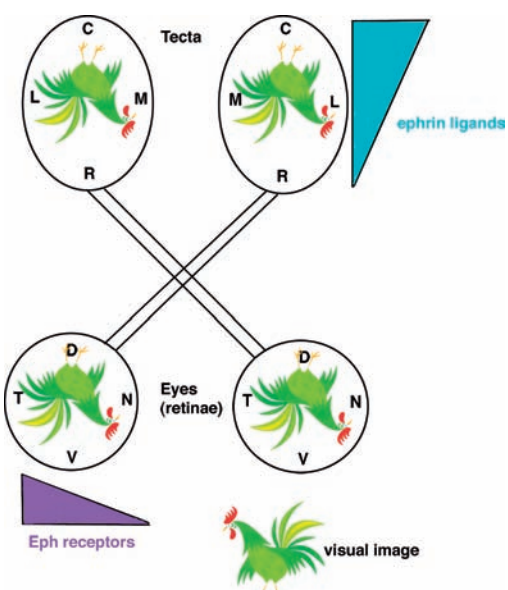
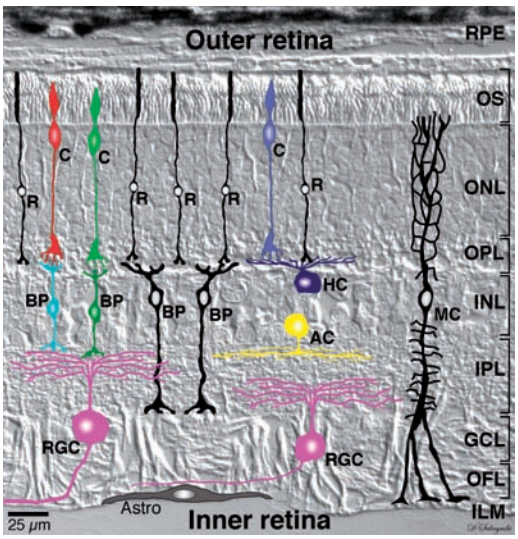


FIGURE 24.6



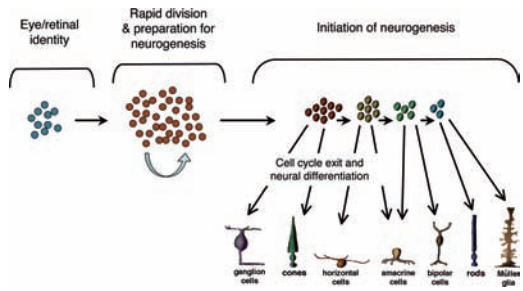


FIGURE 25.5

FIGURE 25.1

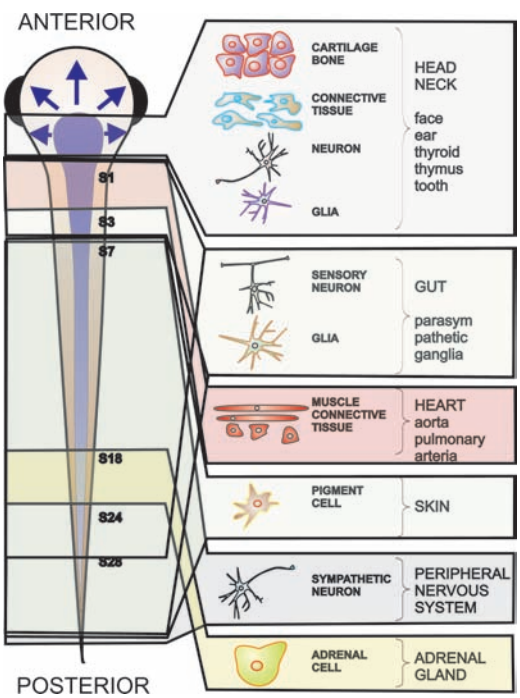


FIGURE 26.2

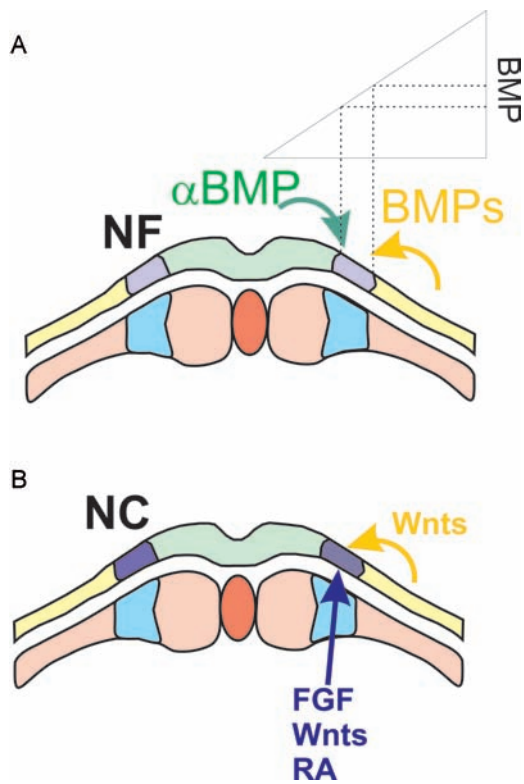


FIGURE 26.4

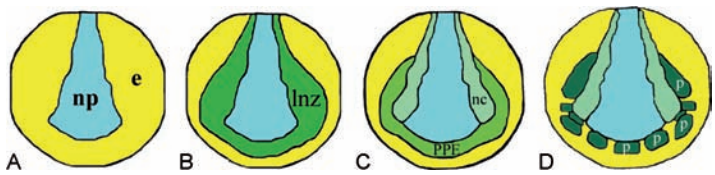


FIGURE 27.1

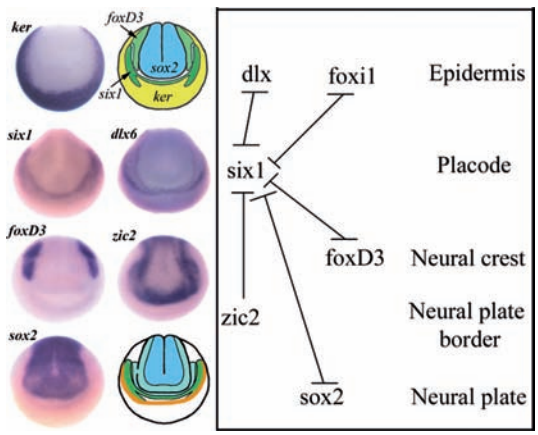


FIGURE 27.4

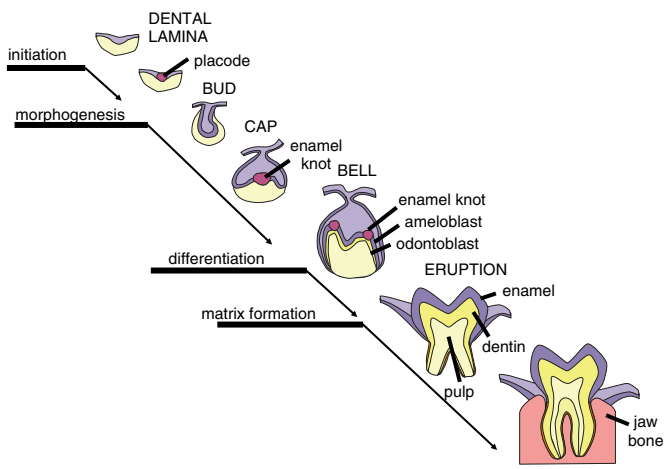


FIGURE 28.1

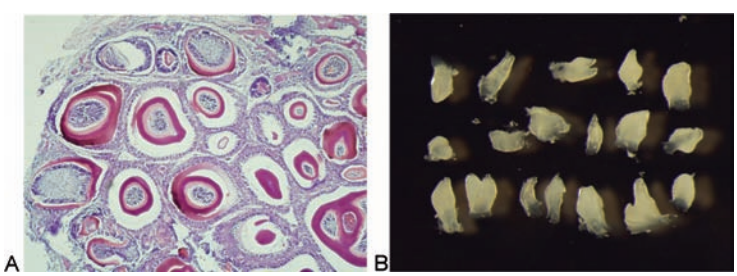


FIGURE 28.5

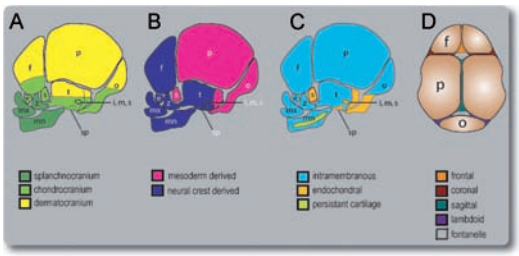


FIGURE 30.1

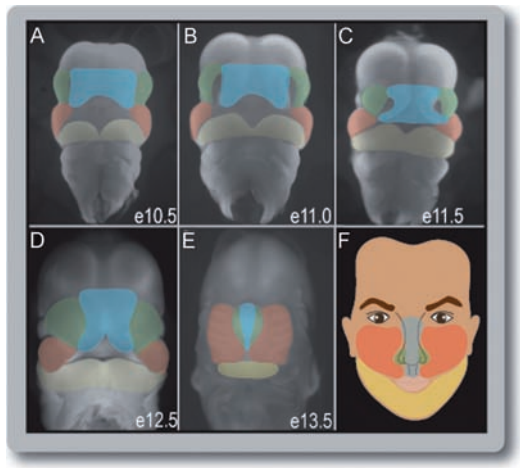


FIGURE 30.2

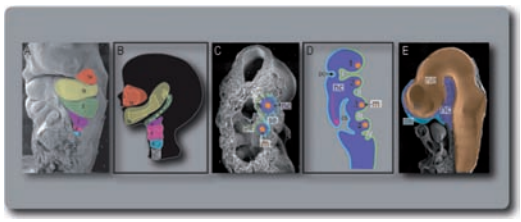


FIGURE 30.4

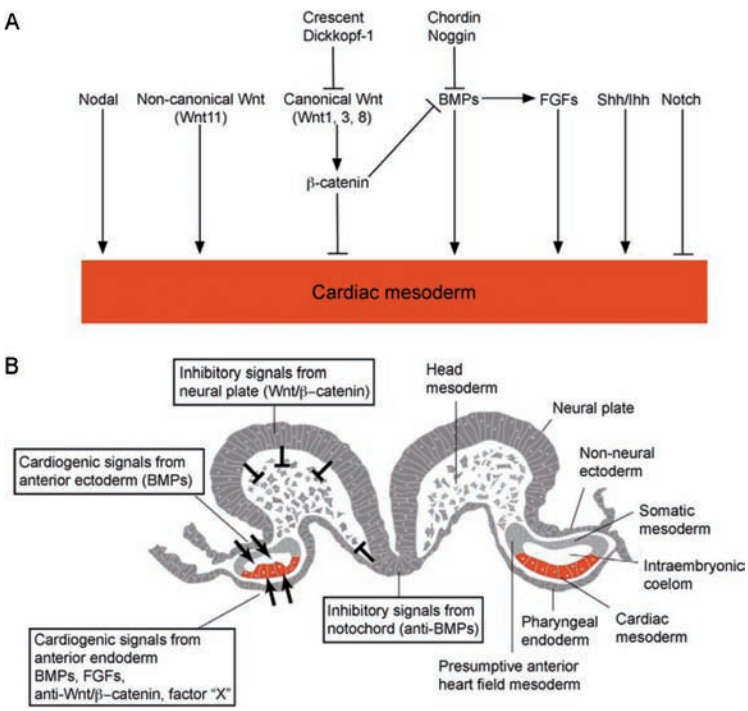


FIGURE 31.2

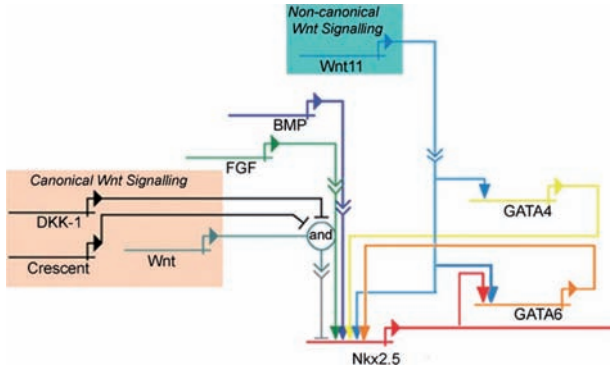


FIGURE 31.3

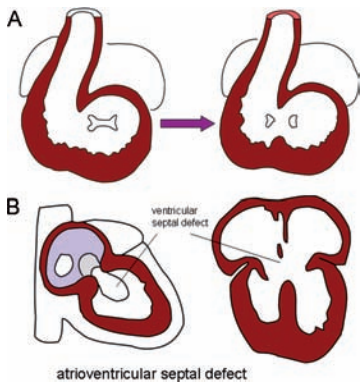


FIGURE 32.2

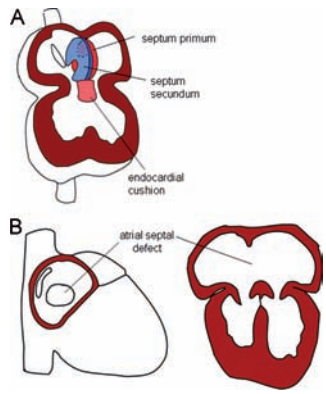


FIGURE 32.3

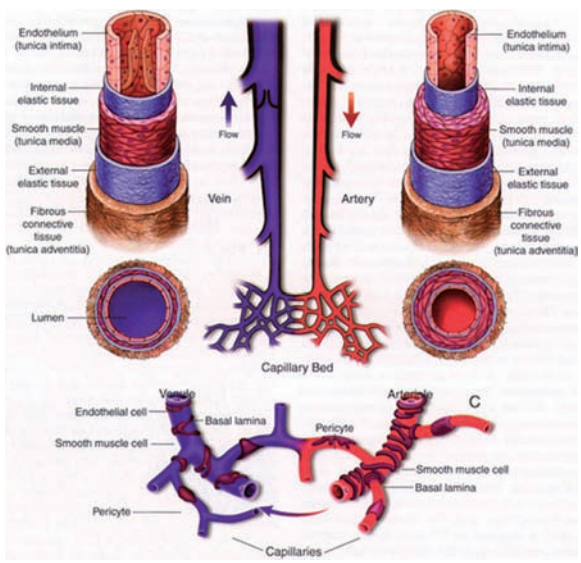


FIGURE 33.1

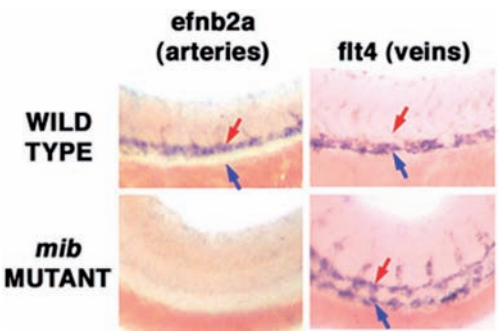


FIGURE 33.3

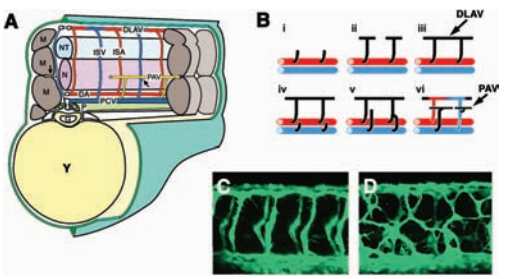


FIGURE 33.6

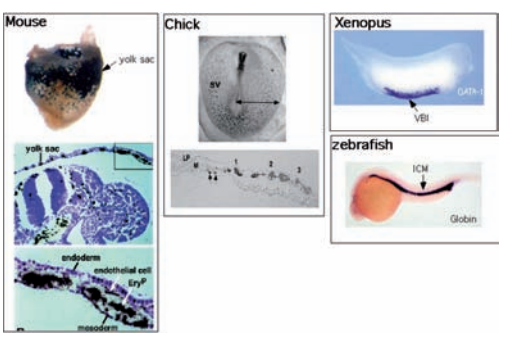


FIGURE 34.2

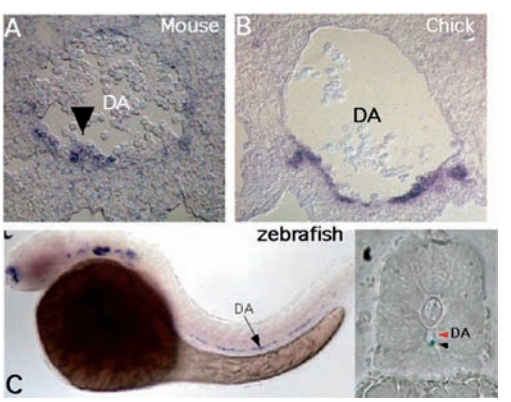


FIGURE 34.3

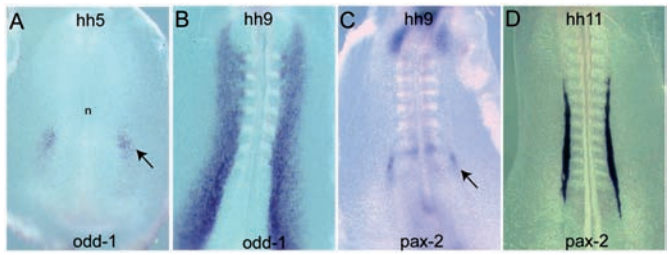


FIGURE 35.6

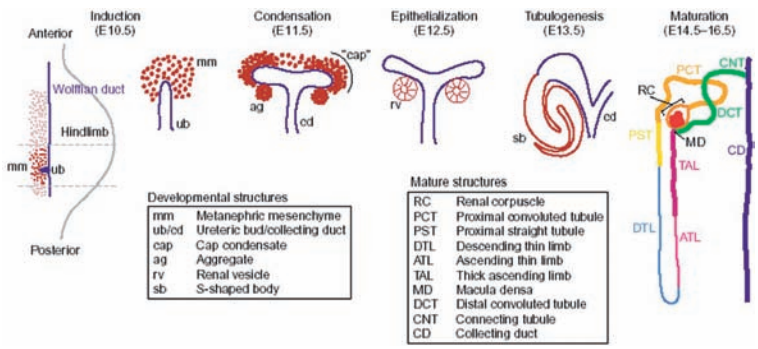
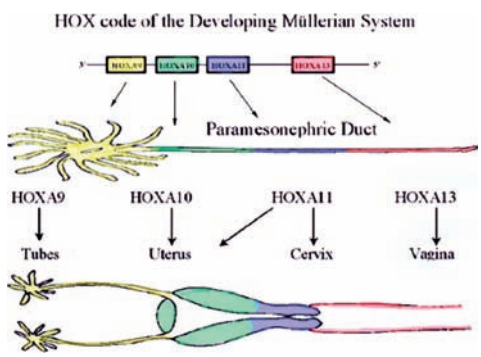


FIGURE 35.8



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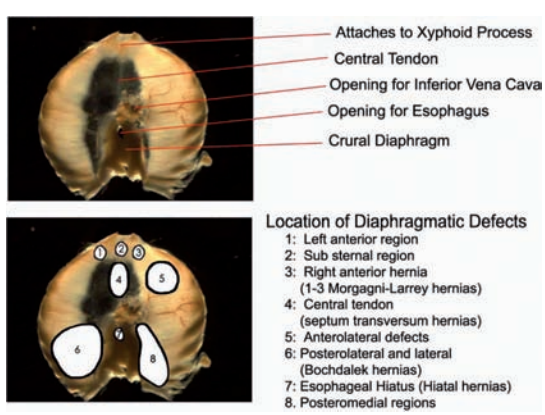
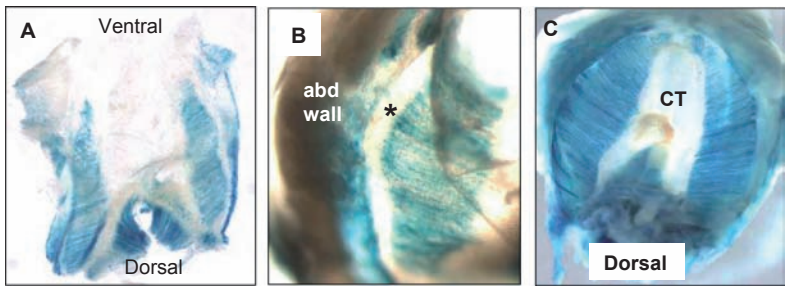


FIGURE 37.1



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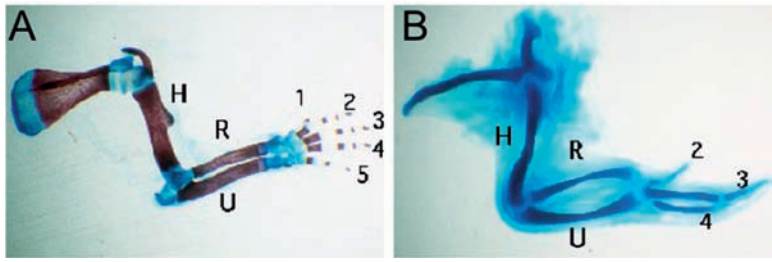

FIGURE 38.2

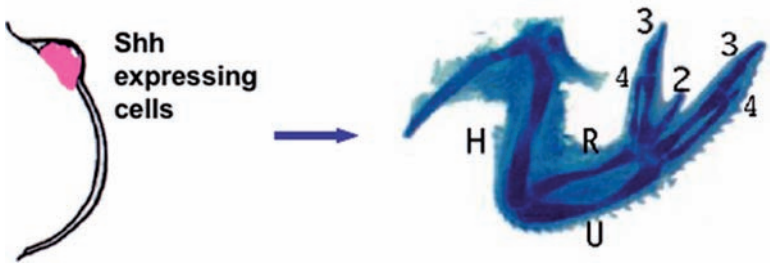

FIGURE 38.3

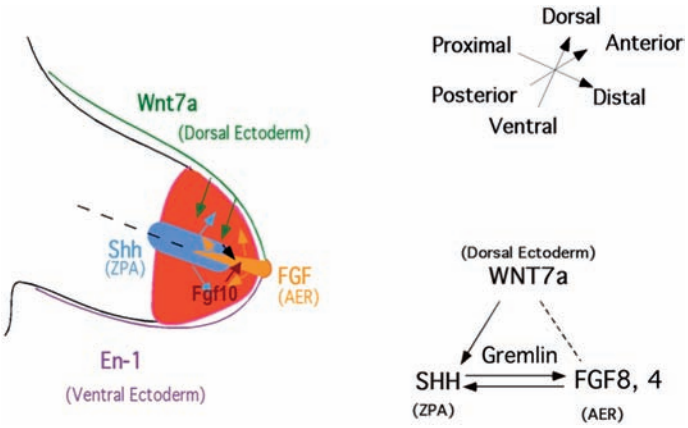

FIGURE 38.5

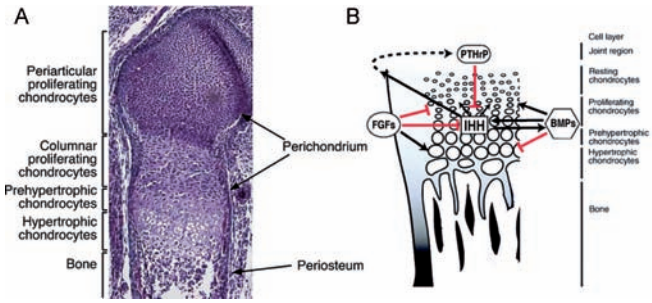

FIGURE 39.2

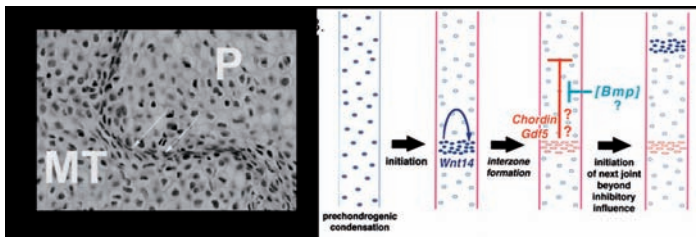

FIGURE 39.3

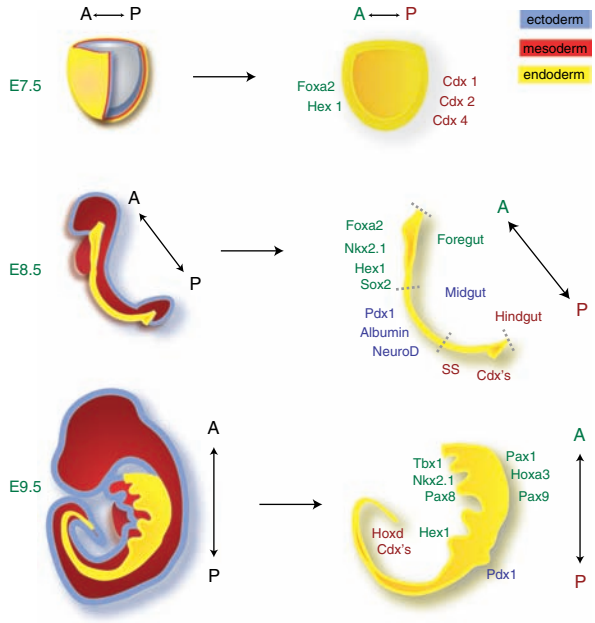

FIGURE 40.1

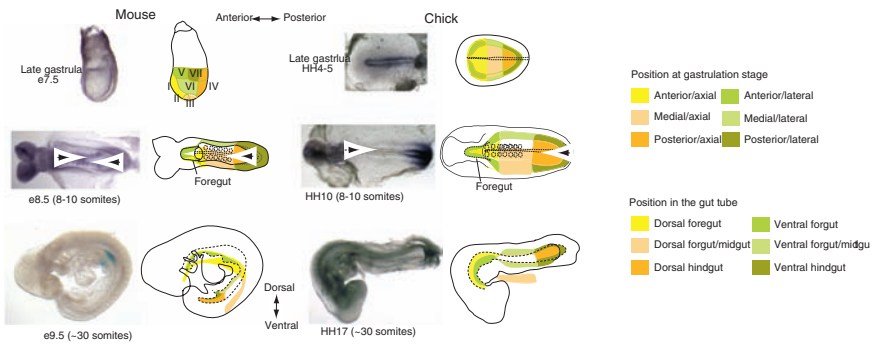

FIGURE 40.3

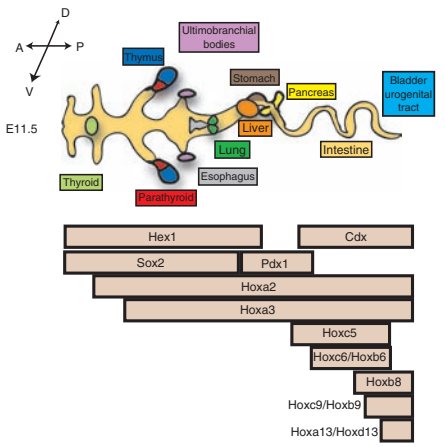

FIGURE 40.4

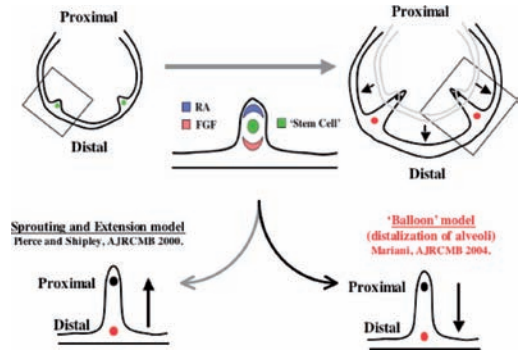

FIGURE 41.2

FIGURE 42.1

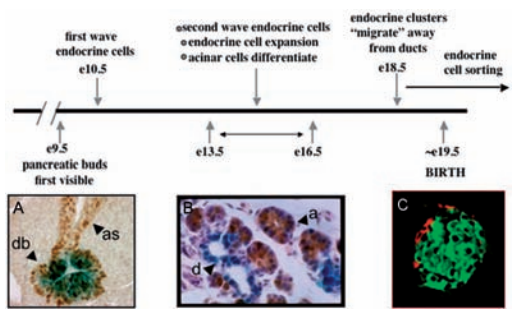

FIGURE 42.2

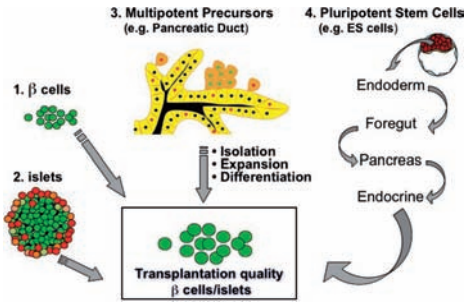

FIGURE 42.4

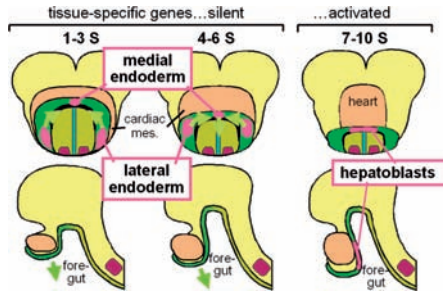

FIGURE 43.1

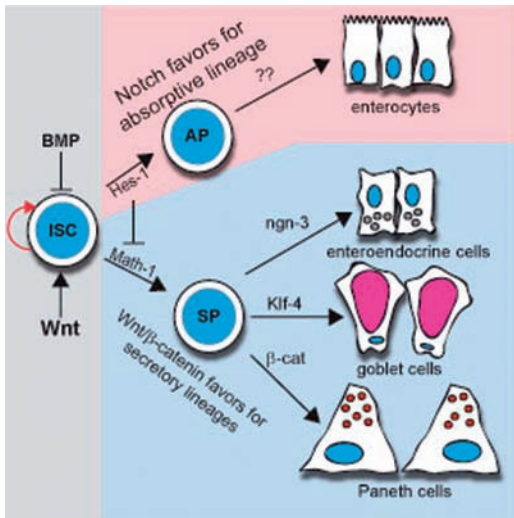

FIGURE 44.3

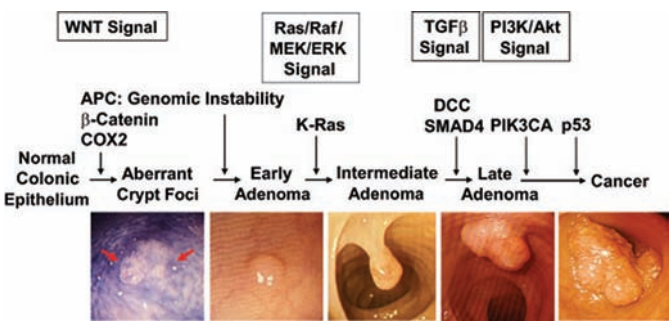

FIGURE 44.4